PB83-241737

Protocol for Bioassessment of Hazardous Waste Sites

Tetra Tech, Inc., Lafayette, CA

Prepared for

Corvallis Environmental Research Lab., OR

Jul 83

U.S. Department of Commerce
National Technical Information Service
NTIS

PB83-241737

EPA-600/2-83-054
July 1983

PROTOCOL FOR BIOASSESSMENT OF HAZARDOUS WASTE SITES

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TC-3547-1

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SPRINGFIELD, VA. 22161

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TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/2-83-054	2.	3. RECIPIENT'S ACCESSION NO. PB83 241737
4. TITLE AND SUBTITLE Protocol for Bioassessment of Hazardous Waste Sites		5. REPORT DATE July 1983
		6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) D.B. Porcella		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Tetra Tech, Inc. 3746 Mt. Diablo Blvd. Lafayette, CE 94549		10. PROGRAM ELEMENT NO.
		11. CONTRACT/GRANT NO. TC-3547-1 PO 2B0177NALX
12. SPONSORING AGENCY NAME AND ADDRESS Environmental Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Corvallis, Oregon 97333		13. TYPE OF REPORT AND PERIOD COVERED project report- final
		14. SPONSORING AGENCY CODE EPA/600/02

15. SUPPLEMENTARY NOTES

Project Officer: W.E. Miller, FTS 420-4669 / S.A. Peterson, FTS 420-4794

16. ABSTRACT

The bioassessment protocol is one of several tools, including chemical analysis and field study, that can be used to characterize the potential environmental risk associated with hazardous waste sites. The protocol can be applied to priority ranking for deciding the need for cleanup of a site compared to other sites, and to assess cleanup effectiveness by testing for potential hazards at the site boundaries or along a sampling transect. Bioassessment involves using defined biological tests to determine the biological response to concentrations of the biologically active components of soil and water samples from a hazardous waste site. The tests are described in the report Appendix and include aquatic and terrestrial tests. The key to defining site priority or cleanup effectiveness is in the experimental sampling design. Careful definition of general and site-specific issues is necessary. The design should be evaluated in terms of cost-benefit so that costly errors in environment risk and economic risk are minimized. Important points about how these concepts relate to sampling design are discussed. The bioassessment protocol is designed to be a set of tools that are applied as appropriate to a specific site. Necessary samples are collected to address the specific issues that occur at the site. Data from chemical analyses and field studies may be available or may be required based on the results obtained from bioassessment.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT Release to public	19. SECURITY CLASS *(This Report)* unclassified	21. NO. OF PAGES 142
	20. SECURITY CLASS *(This page)* unclassified	22. PRICE

EPA Form 2220-1 (9-73)

PREFACE

This bioassessment protocol was developed in steps. First, a set of biological test procedures was defined for possible use. Then, the conceptual basis and the specific tests as related to ecological needs and current regulatory requirements were discussed at a workshop in Washington, D.C. October 26-27, 1981. The attendees also considered the current status of hazardous waste site prioritization and cleanup and statistical factors, field application and evaluation procedures, and other possible biological tests and procedures. The resulting protocol has been extensively reviewed, and is being applied to existing sites to evaluate response levels in relation to field studies. It should be noted that this protocol is not a regulation, rather it is a set of tools for studying potential ecological hazards at waste sites. The protocol relates to the National Contingency Plan (Federal Register, 47(137):31180-31243, July 16, 1982) as published pursuant to Section 311 of the Clean Water Act (CWA) and as revised under Section 105 of the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) of 1980.

ACKNOWLEDGMENTS

The participants in the workshop to discuss this protocol are listed below and represent federal and state agencies, industry, universities, and consultants. This document was prepared and compiled by the author. However, the workshop participants made substantial contributions and subsequently reviewed the document, although they did not necessarily endorse all of its contents. Their efforts are gratefully acknowledged.

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Introductory papers were presented by Peterson (Problem Description), Kleveno (Problem Setting and Site Priority), Gherini (Chemistry), Herricks (Biology), Murarka (Risk Analysis), Miller (Protocol Methods), Porcella (Sampling Criteria), Thomas (Field Studies), and Klein (Microbial Tests). Becky Boone of AWARE, Inc. provided invaluable coordination of the workshop and subsequent mailing of the protocol.

Special thanks go to the Project Officers, Spencer A. Peterson and William A. Miller of the USEPA, Corvallis. They provided overall guidance and constructive criticism. Also, Clarence Callahan, Joseph Greene, and Ibrahim Hindawi, USEPA, critiqued the procedures and approach. In addition to others named, Alan Maki of Exxon and Pat Guiney of Gulf Research reviewed the report.

Steven A. Gherini and Thomas M. Grieb of Tetra Tech, Inc. contributed substantially to the report. However, the author is responsible for any errors. Pencie Shrewsbury prepared the manuscript in its final form. The work was done under a USEPA purchase order, P.O. 2B0177NALX.

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EXECUTIVE SUMMARY

The bioassessment protocol is one of several tools, including chemical analysis and field study, that can be used to characterize the potential environmental risk associated with hazardous waste sites. The protocol can be applied to priority ranking for deciding the need for cleanup of a site compared to other sites, and to assess cleanup effectiveness by testing for potential hazards at the site boundaries or along a sampling transect.

Bioassessment involves using defined biological tests to determine biological response to concentrations of the biologically active components of soil and water samples from a hazardous waste site. The tests are described in Appendix A and include aquatic and terrestrial tests. The algal, fish and *Daphnia* tests are used for water and soil extract samples, and seed germination-root elongation, earthworm, and soil microorganism tests are used for soil samples. The tests are standardized and each has a background of literature citations which include some field evaluation. Because of occupational risks, safe procedures must be used to minimize hazard to staff during field sampling (USEPA, 1981) and during the application of the protocol (Guidelines, Appendix B).

The key to defining site priority or cleanup effectiveness is in the experimental sampling design. Careful definition of general and site-specific issues is necessary. With these issues carefully in mind, the design should be evaluated in terms of cost-benefit so that costly errors in environmental risk and economic risk are minimized. Important points about how these concepts relate to sampling design are discussed in the main text.

The bioassessment protocol is designed to be a set of tools that are applied as appropriate to a specific site. Necessary samples are collected to address the specific issues that occur at the site. Data from chemical analyses and field studies may be available or may be required based on the results obtained from bioassessment.

The bioassessment protocol will be improved for future use with field application and with further research. It is promulgated at this time because there is a present need for such a protocol, and ongoing field application will lead directly to its improvement.

INTRODUCTION

ASSESSING WASTE DISPOSAL SITES

The potential hazard of planned, existing or abandoned waste disposal sites depends on their risk to human health and the environment. Generally, these hazards fall into four categories: toxicity, persistence, bioaccumulation, and mobility. To minimize these hazards, cleanup and control actions are taken based on data describing site characteristics.

The identification, characterization, and cleanup of hazardous materials, sites and spills is a high priority of the U.S. Environmental Protection Agency and society. Numerous potential cleanup sites have been identified throughout the United States, and 418 priority sites have been selected for cleanup (HMIR, 1981). Further characterization and cleanup of approximately a dozen of the worst sites is expected to proceed shortly. However it is not entirely clear how these characterizations and cleanups will be conducted. The National Contingency Plan indicated that chemical characterization of sites would have a high priority. It was proposed that existing water and air quality criteria would be applied to chemical characterizations to determine when cleanup was necessary, how much to cleanup, and when to terminate the cleanup.

This approach has been criticized for various reasons. Among the reasons is that criteria applications would tend to be overly protective and thus overly restrictive. Another criticism is that insufficient numbers of adequate criteria exist. Yet another criticism is that single pollutant, constant concentration, laboratory derived criteria are not applicable to environmentally released complex hazardous waste materials which may be encountered at numerous disposal sites. If water and air quality criteria are not applied then, what tools will be employed to assess the need for and degree of cleanup required?

The multi-media biological testing protocol presented in this document is an alternative or supplement to other assessment techniques. It is not without problems, but currently it is felt that the advantages outweigh the disadvantages.

The overall objective of this report is to describe a bioassessment protocol that can be used immediately while being tested over a broad range of pollutant and geoclimatic conditions. The protocol consists of sampling environmental media followed by the application of specific biological tests. The protocol is intended to be applied to existing or abandoned waste disposal sites, but at least some of the procedures can be applied during planning of new sites, to spills, or to new chemicals and materials under appropriate conditions. Application of the data gained from these tests will be on a case by case basis depending on the potential for environmental impact and the intended use of the area in question.

The bioassessment protocol consists of a set of specifically defined biological tests, and incorporate sampling design and statistical analysis concepts. The protocol includes short term, acute tests for toxicity using aquatic and terrestrial organisms. Except for the soil litter test, the biological tests utilize only a single species but they do include plants, invertebrates, vertebrates, and decomposers. The biological tests are presented in a stepwise manner in Appendix A while the protocol is presented in the text.

PURPOSE OF THE BIOASSESSMENT PROTOCOL

The bioassessment protocol has the purpose of assessing the potential for ecological harm from hazardous waste sites, and is one set of tools, along with chemical and field studies, that can be used to minimize the risk from hazardous materials. The responses of a range of test species to exposure from water and soil samples are used to determine whether toxicity exists at a site. The biological tests that make up the protocol cover a range of biological taxa, are standardized, and have a history of use for a variety of environmental assessments.

The bioassessment protocol is used for two purposes: site prioritization and cleanup evaluation. Depending on a variety of factors including concentration, type and availability of chemicals, organisms at risk, exposure routes, and duration of exposure, certain sites have the potential for more or less ecological risk. By incorporating these risk factors, the test results can be used to rank sites in order of priority for cleanup, isolation, or other action. Cleanup evaluation is the application of the biological tests to determine: "How clean is a site?" States, industries, and federal agencies will be interested in using these tests for these purposes.

A BIOASSESSMENT ANALOGY

The ecosystem is an entity in which humanity and society are an integral part. Protecting natural communities will, in most cases, afford protection for human communities. The state of the natural community can be viewed as an indicator of potential risk to society and effects on important processes can be viewed as proxies for those processes that directly as well as indirectly relate to society. The bioassessment protocol can be viewed as a quantitative means of estimating biological impacts of hazardous waste sites. The individual tests are bio-transducers for environmental protection, providing an estimate of potential hazard to organisms caused by chemicals that vary in their availability and toxicity in water or soil.

The potential for damage to human health and the ecological integrity of hazardous waste sites can be perceived similarly to a dying canary in a coal mine. The analogy applies to this protocol because bioassessment methods are intended to provide a measure of potential acute biological damage associated with samples from a particular site. A canary breathes the mine air, responds to all biologically active components of the atmosphere, and if an acute response occurs, dies, at which time the miners flee. If the canary sickens over a long period of time, it would be replaced and probably no human response would occur. Thus, chronic effects are not assessed.

The bioassessment procedures are similar to the canary in the mine and provide a rapid screening of all of the biologically active components of hazardous waste sites, and if an acute response is obtained, provide a signal causing an appropriate response by society. The key points are:

- Bioassessment provides a biological response which integrates all of the biologically active components in a sample.

- Bioassessment provides an estimate of the biologically available forms of the sample components.

PROTOCOL OBJECTIVES

The overall objective of the bioassessment protocol is to provide a more comprehensive measure of potential ecological hazard associated with hazardous waste sites than chemical analyses and comparison to air and water quality criteria can provide by themselves. Also, a preliminary assessment can be obtained about sites where few data are available. Proper application of the biological tests can improve the accuracy of the assessment as well as improve cost-effectiveness by prioritizing hazardous sites. Specific steps must be followed to achieve these objectives:

- Identify the waste disposal site.

- Define potential transport and fate of site materials and populations at risk; list risk issues of concern.

- Define containment site boundaries.

- Design a sampling program to meet specified statistical criteria.

- Obtain appropriate soil and surface and ground water samples at the site boundary.

- Obtain appropriate soil and surface and ground water samples along a gradient of waste contamination.

- Select biological tests appropriate to answering the risk issues defined previously.

- Perform necessary pretreatment of samples and the biological tests.

Some of these steps may be repeated based on biological test results. For example, containment site boundaries might be extended because protocol results show that soil and ground water samples beyond the defined boundary are excessively contaminated.

BIOASSESSMENT PROTOCOL

PERSPECTIVE

Hazardous wastes have been characterized as wastes from a list of specific industries (Table 1), wastes containing one or more components on the priority pollutants list (Table 2), or a waste that is ignitable, corrosive, reactive, or toxic. Also, wastes are hazardous if they exhibit chemical measurements of a specified leachate that exceed the national drinking water standards by a factor of 100 or more (Table 3). Concepts and controversy relating to the characterization of hazardous wastes have been discussed elsewhere (Anonymous, 1981). The tests described in this protocol will be useful in refining these definitions.

To gain more perspective on the purpose of bioassessment, it is instructive to ask which potential hazards are not being assessed with biological tests. The bioassessment protocol only addresses the acute toxicity issue. In addition, volatile materials will probably not be assessed accurately. The problem of bioaccumulation is not evaluated at present. Thus, research is needed to develop procedures to evaluate volatile materials and bioaccumulation. Chronic toxicity, carcinogenesis, mutagenesis, and teratogenesis are not assessed using the bioassessment protocol. A Level 2 protocol could be defined for assessing these hazards.

Although the bioassessment protocol does not measure all facets of hazardous waste site problems, it does provide a measure of those factors that directly affect environmental processes, i.e. acute toxicity. In this

Table 1

EPA PRIMARY INDUSTRY CATEGORIES

Adhesives and sealants
Aluminum forming
Auto and other laundries
Battery manufacturing
Coal mining
Coil coating
Copper forming
Electric and electronic components
Electroplating
Explosives manufacturing
Foundries
Gum and wood chemicals
Inorganic chemicals manufacturing
Iron and steel manufacturing
Leather tanning and finishing
Mechanical products manufacturing
Nonferrous metals manufacturing
Ore mining
Organic chemicals manufacturing
Paint and ink formulation
Pesticides
Petroleum refining
Pharmaceutical preparations
Photographic equipment and supplies
Plastic and synthetic materials manufacturing
Plastic processing
Porcelain enameling
Printing and publishing
Pulp and paperboard mills
Rubber processing
Soap and detergent manufacturing
Steam electric power plants
Textile mills
Timber products processing

Table 2

EPA'S PRIORITY TOXIC POLLUTANTS

1. *acenaphthene
2. *acrolein
3. *acrylonitrile
4. *benzene
5. *benzidine
6. *carbon tetrachloride (tetrachloromethane)

*chlorinated benzenes (other than dichlorobenzienes)

7. chlorobenzene
8. 1,2,3-trichlorobeinzene
9. hexachlorobenzene

*chlorinated ethanes (including 1,2-dichloroethane, 1,1,1-trichloroethane and hexachloroethane)

10. 1,2-dichloroethane
11. 1,1,1-trichloroethane
12. hexachloroethane
13. 1,1-dichloroethane
14. 1,1,2-trichloroethane
15. 1,1,2,2-tetrachloroethane
16. chloroethane

*chloroalkyl ethers (chloromethyl, chloroethyl and mixed ethers)

17. bis(chloromethyl) ether
18. bis(2-chloroethyl) ether
19. 2-chloroethyl vinyl ether (mixed)

*chlorinated naphthalene

20. 2-chloronaphthalene

*chlorinated phenols (other than those listed elsewhere; includes trichlorophenols and chlorinated cresols)

21. 2,4,6-trichlorophenol
22. parachlorometa cresol
23. *chloroform (trichloromethane)
24. *2-chlorophenol

*dichlorobenzenes

25. 1,2-dichlorobenzene
26. 1,3-dichlorobenzene
27. 1,4-dichlorobenzene

*dichlorobenzidine

28. 3,3-dichlorobenzidine

*dichloroethylenes (1,1-dichloroethylene and 1,2-dichloroethylene)

29. 1,1-dichloroethylene
30. 1,2-trans-dichloroethylene
31. *1,3-dichlorophenol

*dichloropropane and dichloropropene

32. 1,2-dichloropropane
33. 1,2-dichloropropylene (1,3-dichloropropene)
34. *2,4-dimethylphenol

*dinitrotoluene

35. 2,4-dinitrotoluene
36. 2,6-dinitrotoluene
37. *1,2-diphenylhydrazine
38. *ethylbenzene
39. *fluoranthene

*haloethers (other than those listed elsewhere)

40. 4-chlorophenyl phenyl ether
41. 4-bromophenyl phenyl ether
42. bis(2-chloroisopropyl) ether
43. bis(2-chloroethoxy) methane

*halomethanes (other than those listed elsewhere)

44. methylene chloride (dichloromethane)
45. methyl chloride (chloromethane)
46. methyl bromide (bromomethane)
47. bromoform (tribromomethane)
48. dichlorobromomethane
49. trichlorofluoromethane
50. dichlorodifluoromethane
51. chlorodibromomethane
52. *hexachlorobutadiene
53. *hexachlorocyclopentadiene
54. *isophorone
55. *naphthalene
56. *nitrobenzene

*nitrophenols (including 2,4-dinitrophenol and dinitrocresol)

57. 2-nitrophenol
58. 4-nitrophenol
59. 2,4-dinitrophenol
60. 4,6-dinitro-o-cresol

*nitrosamines

61. N-nitrosodimethylamine
62. N-nitrosodiphenylamine
63. N-nitrosodi-n-propylamine
64. *pentachlorophenol
65. *phenol

*phthalate esters

66. bis(2-ethylhexyl) phthalate
67. butyl benzyl phthalate
68. di-n-butyl phthalate
69. di-n-octyl phthalate
70. diethyl phthalate
71. dimethyl phthalate

*polynuclear aromatic hydrocarbons

72. benzo(a)anthracene (1,2-benzanthracene)
73. benzo(a)pyrene (3,4-benzopyrene)
74. 3,4-benzofluoranthene
75. benzo(k)fluoranthane (11,12-benzofluoranthene
76. chrysene
77. acenaphthylene
78. anthracene
79. benzo(ghi)perylene (1,12-benzoperylene)
80. fluorene
81. phenanthrene
82. dibenzo (a,h) anthracene (1,2,5,6-dibenzanthracene)
83. indeno (1,2,3-cd)pyrene (2,3-o-phenylenepyrene)
84. pyrene
85. *tetrachloroethylene
86. *toluene
87. *trichloroethylene
88. *vinyl chloride (chloroethylene)

pesticides and metabolites

89. *aldrin
90. *dieldrin
91. *chlordane (technical mixture & metabolites)

*DDT and metabolites

92. 4,4'-DDT
93. 4,4'-DDE (p,p'-DDX)
94. 4,4'-DDD (p,p'-TDE)

*endosulfan and metabolites

95. a-endosulfan-Alpha
96. b-endosulfan-Beta
97. endosulfan sulfate

*endrin and metabolites

98. endrin
99. endrin aldehyde

*heptachlor and metabolites

100. heptachlor
101. heptachlor epoxide

*hexachlorocyclohexane (all isomers)

102. a-BHC-Alpha
103. b-BHC-Beta
104. r-BHC (lindane) -Gamma
105. g-BHC-Delta

*polychlorinated biphenyls (PCB's)

106. PCB-1242 (Arochlor 1242)
107. PCB-1254 (Arochlor 1254)
108. PCB-1221 (Arochlor 1221)
109. PCB-1232 (Arochlor 1232)
110. PCB-1248 (Arochlor 1248)
111. PCB-1260 (Arochlor 1260)
112. PCB-1016 (Arochlor 1016)
113. *toxaphene
114. *antimony (total)
115. *arsenic (total)
116. *asbestos (fibrous)
117. *beryllium (total)
118. *cadmium (total)
119. *chromium (total)
120. *copper (total)
121. *cyanide (total)
122. *lead (total)
123. *mercury (total)
124. *nickel (total)
125. *selenium (total)
126. *silver (total)
127. *thallium (total)
128. *zinc (total)
129. **2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

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* Specific compounds and chemical classes listed in the NRDC consent decree and referenced in the Clean Water Act.

** This compound was specifically listed in the consent decree; however, due to its extreme toxicity EPA recommends that laboratories not acquire an analytical standard for this compound.

Table 3

PRIMARY DRINKING WATER QUALITY STANDARDS

Parameters	Annual Average Maximum Daily Air Temperature °F	°C	Maximum Level[a]
Inorganic Chemicals			
Arsenic			0.05
Barium			1.
Cadmium			0.010
Chromium			0.05
Lead			0.05
Mercury			0.002
Nitrate (as N)			10.
Selenium			0.01
Silver			0.05
Fluoride			
	53.7 and below	12.0 and below	2.4
	53.8 to 58.3	12.1 to 14.6	2.2
	58.4 to 63.8	14.7 to 17.6	2.0
	63.9 to 70.6	17.7 to 21.4	1.8
	70.7 to 79.2	21.5 to 26.2	1.6
	79.3 to 90.5	26.3 to 32.5	1.4
Chlorinated Hydrocarbons			
Endrin (1, 2, 3, 4, 10, 10-hexachloro-6, 7-epoxy-1, 4, 4a, 5 6, 7, 8, 8a-octahydro-1, 4-endo-5, 8-dimethano naphthalene)			0.0002
Lindane (1, 2, 3, 4, 5, 6-hexachlorocyclohexane, gamma isomer)			0.004
Methoxychlor (1, 1, 1-Trichloroethane) 1, 1-bis (p-methoxyphenyl)			0.1
Toxaphene ($C_{10}H_{10}Cl_8$-Technical chlorinated camphene, 67-68 percent chlorine)			0.005
Chlorophenoxys: 2,4-D, (2, 4-Dichlorophenoxyacetic acid) 2, 4, 5-TP Silvex (2, 4, 5-Trichlorophenoxy-propionic acid)			0.1 0.01
Turbidity (for surface water sources)	1 TU up to 5 TU[b]		
Coliform Bacteria			
Membrane filter technique:	1/100 ml mean/month 4/100 ml in one sample if <20 samples/month 4/100 ml in more than 5% if >20 samples/month		
Fermentation tube with 10 ml portions:	no coliforms in 10% of portions/month no coliforms in >3 portions/sample if <20 samples/month no coliforms in >3 portions of 5% of samples if >20 samples/month		
Fermentation tube with 100 ml portions:	no coliform bacteria in >60% of portions/month no coliform in 5 portions in one sample if <5 samples/month no coliform in 5 portions in 20% of samples if >5 samples/month		
Radioactive Material			**Level**
Combined radium 226 and radium 228			5 pCi/l
Gross alpha particle activity[c]			15 pCi/l
Beta particle and photon radioactivity from man-made radionuclides			4 millirem/year
Tritium for total body			20,000 pCi/l
Strontium-90 in bone marrow			8 pCi/l

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[a] mg/l unless otherwise stated.

[b] If meet special requirement.

[c] Includes Ra^{226} excludes Radon, Uranium.

Source: EPA (1977)

regard the biological tests are useful for assessing the potential ecological hazard of hazardous wastes. Ignitability, corrosivity and reactivity have impacts that are catastrophic and overshadow long-range environmental concerns, and therefore, require more immediate action. However, the bioassessment tests provide responses to the toxicity of such materials.

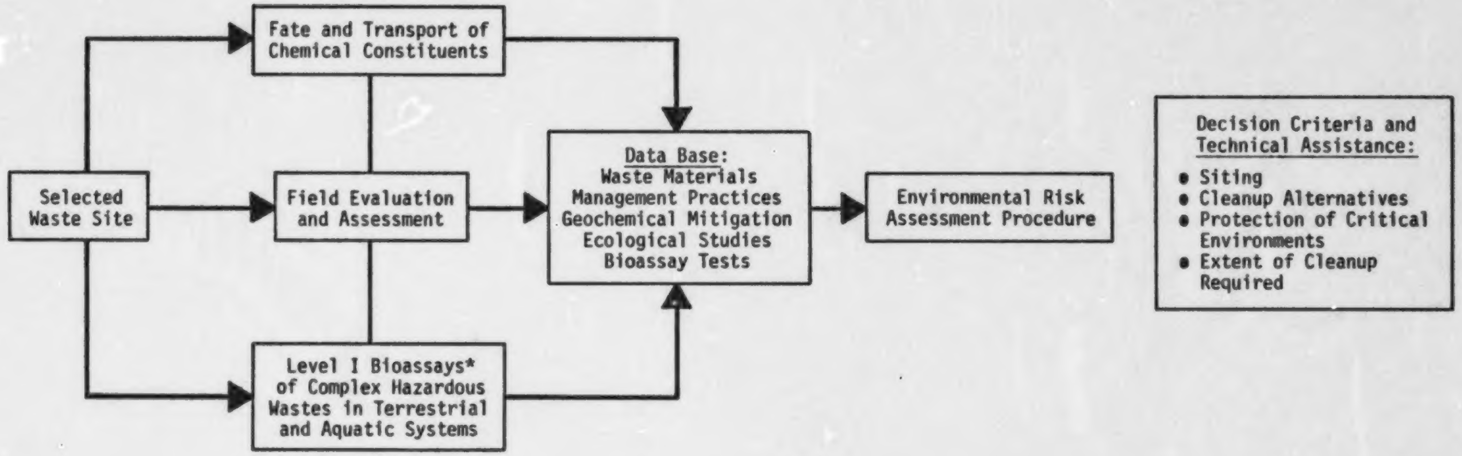
An accurate perspective on bioassessment tests requires consideration of the role of chemical analysis and field ecological studies in evaluating hazardous waste sites. Chemical analysis is important for actual cleanup procedures and for evaluating the fate and effects of materials from hazardous waste sites. An extensive list of chemicals such as those in Table 2 and 3 can be measured in water, soil, and soil leachate samples. The measured concentrations can be compared to standards or criteria extrapolated from laboratory bioassay procedures. Then, appropriate actions are taken according to published regulations; however, considerable uncertainty remains. The decision maker is never certain whether all toxic chemicals are on the list, whether they are measured adequately, or whether the mixture exerts different toxicity than the sum of the individual chemicals. Also, not all of the chemicals are equally available to organisms under a particular set of conditions. Although chemical results are administratively easy to use, they are not as meaningful in an ecological context as biological measurements. Although the relative precision of biological tests compared to chemical tests is often questioned, biological tests have been found to be as precise as chemical tests if proper procedures are followed. Moreover, the question of precision is less important than accuracy, that is, integrating the effects and bioavailability of compounds to organisms.

The most direct approach for evaluating the ecological hazard of an hazardous waste site is to observe effects on the ecosystem by field studies. However, several disadvantages for this approach exist. To observe effects, damage to the ecosystem must have occurred already, and that is what should be avoided. In addition, field ecological studies are often expensive and time consuming, are not predictive and in fact are retrospective. On the other hand, field studies are necessary for many purposes because they directly measure ecological harm.

We conclude from the above discussion that biological tests are necessary for evaluating potential ecological effects of hazardous waste sites. However, the biological tests must undergo testing by comparing biological test results to chemical and field study data. This process is being conducted by the U.S. EPA, Corvallis Environmental Research Laboratory, Oregon (Figure 1). It is expected that others will use the tests and report their results. As experience is gained in applying the bioassessment protocol, it will be improved.

For regulatory reasons, the bioassessment protocol cannot supplant all chemical and field data. Many of these data will be available at the initiation of site studies or are obtained by observation and measurement at that time. Coordination of this information with the bioassessment protocol results is an important need in protocol development.

As an example of how the bioassessment protocol can be incorporated into assessment of a hazardous waste site, we have included excerpts from a site response management plan from USEPA's Region IV. The following discussion is taken directly from Mathis (1981) with minor modification


Selected Waste Site
Fate and Transport of Chemical Constituents
Field Evaluation and Assessment
Level I Bioassays* of Complex Hazardous Wastes in Terrestrial and Aquatic Systems
Data Base:
Waste Materials
Management Practices
Geochemical Mitigation
Ecological Studies
Bioassay Tests
Environmental Risk Assessment Procedure
Decision Criteria and Technical Assistance:
Siting
Cleanup Alternatives
Protection of Critical Environments
Extent of Cleanup Required

*Application of Bioassessment Protocol.

Figure 1. Evaluation of Environmental Testing Protocols for Hazardous Waste Assessment (Peterson, 1982)

including provision for applying the bioassessment protocol. The EPA Site Tracking System is portrayed in a flow chart (Figure 2). Circled numbers in the flow chart correspond approximately to the numbered sections in the text.

A principal investigator with responsibility for site evaluation is assigned. Typically, the major milestones in a site evaluation are as follows:

1. SITE IDENTIFICATION This represents the entry of a potential uncontrolled site into the system, and may be initiated through any of the many methods of information gathering. General guidance is that in the absence of an affirmative showing that no hazardous material is involved, all reported potential sites will be entered into the system.

2. PRELIMINARY ASSESSMENT At this point the investigator completes a search of available files in federal and state agencies, usually accomplished by telephone contact outside the agency, and will also complete telephone interviews with identified persons having knowledge of this site. The purpose of this action is to discern possible releases which would require emergency containment action to mitigate an imminent hazard to health or the environment, with immediate investigation (response time in hours); and to permit a subjective evaluation of the degree of hazard of other sites as HIGH MEDIUM, LOW, NONE, or UNKNOWN. This judgment becomes the basis for prioritizing the site for the next level of investigation. Resources expended at this point generally range from 0.5 to 1.5 person-days.

3. SITE INSPECTION This activity involves a visit to the site by a team of at least two investigators. Their function is to observe the potential site prior to entry, assess risk for site entry, interview knowledgeable indigenous personnel, appraise the population at risk, identify potential exposure routes, and if justified, enter the site to observe and subjectively evaluate topography, geology, quantity and type of material present, conditions of storage or disposal, evidence or probability of release or migration, and resources needed to quantify or objectively measure these parameters. Due to safety considerations, no sampling is conducted at this time; however, considerable information is collected and recorded in the form of observations and photographs. Resources expended in this activity range from 2.0 to 4.0 work-days exclusive of travel. The purpose of this activity is to produce a more certain evaluation of the potential hazard as HIGH, MEDIUM, LOW, or NONE, to prioritize the site for field investigation as needed and to permit preparation of a TENTATIVE DISPOSITION.

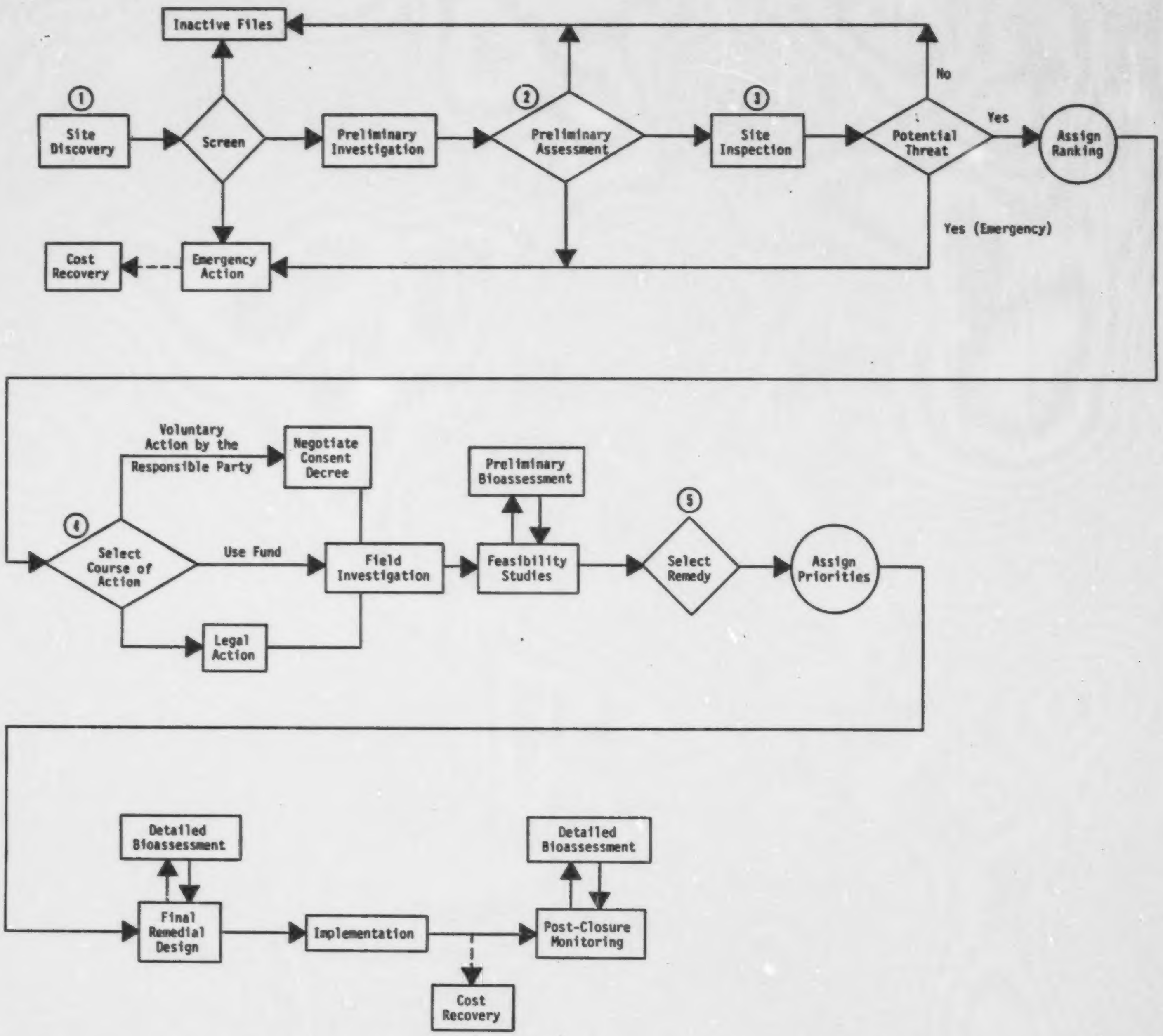

①
Site Discovery
Screen
Inactive Files
Emergency Action
Cost Recovery
Preliminary Investigation
②
Preliminary Assessment
③
Site Inspection
Potential Threat
No
Yes
Yes (Emergency)
Assign Ranking
④
Select Course of Action
Voluntary Action by the Responsible Party
Negotiate Consent Decree
Use Fund
Field Investigation
Legal Action
Preliminary Bioassessment
Feasibility Studies
⑤
Select Remedy
Assign Priorities
Detailed Bioassessment
Final Remedial Design
Implementation
Cost Recovery
Detailed Bioassessment
Post-Closure Monitoring

Figure 2. Site Response Management Plan (from Mathis, 1981). Circled numbers Approximately Correspond to Text Numbers.

4. TENTATIVE DISPOSITION This activity comprises a decision point from which the site is tentatively classified as most appropriate for one of four courses of action. The decision is recommended by the principal investigator, and is reviewed by appropriate section leaders for concurrance. Basis for this recommended decision is a review of all assembled information on the site. Resources required are approximately 1.0 work-days plus review. The four alternative decisions possible are:

   a. Enforcement Action by State or Federal Agency.
      This implies that a viable defendant and an imminent hazard are both present.
   b. Remedial Action using Federal, State or Other Resources.
      This implies that either a responsible party may be willing to undertake necessary action, or that no viable defendant is apparent and direct action using the authority of CERCLA seems appropriate. It also implies that an imminent hazard is present.
   c. Further Investigation Needed.
      This indicates that collection of field data through sampling, geophysical studies, the bioassessment protocol, or other means is required to ascertain the presence or absence of an imminent hazard, or to quantify and delineate the extent of that hazard. This mandates a resource-intensive investigation, and requires a review to set the priority for this effort. Prioritization may be aided by an initial preliminary assessment using the bioassessment protocol.
   d. No Further Action Required.
      This implies that uncontrolled hazardous material is not present at this time, and no significant hazard exists.

5. FINAL STRATEGY DETERMINATION This activity represents the coordinated timetable for a recommended Enforcement Action or Remedial Action, the timetable for the required investigations, or the final concurrance in a finding of No Further Action Required. Final Strategy Determinations are tracked, and are amended as progress is made on the respective timetable. When a Final Strategy Determination of No Further Action is reached the site may be placed in the inactive file of the system. Resource requirements in this stage are highly variable. The effectiveness of cleanup procedures can be evaluated using the bioassessment protocol. It is possible that litigation may take several years to complete, at great cost, and remedial measures may entail costs in the millions. Extensive investigation effort may cost hundreds of thousands of dollars and involve many months of effort. In contrast, a determination of no further action may be processed in a single work-day.

It is noteworthy that at each milestone in the process there is an opportunity to reappraise the priority of each site for the next level of activity, and that at each milestone, limited resources are focused on those sites which are most significant in terms of:

1. Seriousness of hazard to health.
2. Seriousness of hazard to the environment.
3. Presence of a viable defendant or responsible party.
4. Existence of a technically feasible remedy.
5. Availability of uncommitted and appropriate resources.
6. Other factors which tend to raise priority; eg, State Opinion.

Generally, as a high priority site advances through the process, increasing amounts of money and resources are required. This situation has resulted in the identification of several rate-limiting steps in the processing of potential uncontrolled hazardous material sites.

The first of these limitations is the availability of analytical support for the program. Analytical capability, and the associated quality assurance support, is a finite resource due to the high cost, and is also finite in terms of physical capacity. Furthermore, the pursuit of Enforcement Options places an even greater demand on this resource than investigation or remedial design, due to the need for elaborate amounts of data of unimpeachable quality. It may also be projected that where remedial actions are conducted in anticipation of a possible legal action to recover costs, documenting the findings, the action taken, and the benefits accrued thereby, will all necessitate a significant increase in analytical effort beyond that minimum necessary to design and implement the remedy. Proper application of the bioassessment protocol may allow better definition of the priority of a site and thus minimize the number of sites requiring such detail.

The second limitation which has emerged is the need for intensive expenditures of manpower to perform on-site tasks in a safe manner. To approach a totally unknown site in what is generally agreed to be a conservative, safety conscious method requires a team of 5 to 6 persons in order to cope with all contingencies. Furthermore, this team requires elaborate and costly equipment to detect possible agents in real time, while providing protection against a wide spectrum of toxicants. This equipment, in turn, requires recurrent training of personnel and periodic maintenance if it is to be used effectively. The bioassessment protocol could provide an excellent solution for this problem by showing those sites and/or locations where acute biological risks are present.

Finally, the team and its special equipment are not readily transportable by many commercial carriers when prepared for operation. While many regulatory agencies and private laboratories acknowledge similar safety criteria for site investigation, very few of them are staffed or funded to allow operation in accordance with recommended procedures. Thus, specialized contractors may often be the most feasible choice for site sampling. Although the bioassessment protocol may not be directly applicable here, it can be used to minimize needs for outside contractors by a prioritization of sites based on biological responses.

APPLICATION OF BIOASSESSMENT PROTOCOL

At least three scenarios regarding hazardous waste sites in relation to the potential application of bioassessment can be defined:

- The site is uncharacterized and unprioritized. In this case an assessment of potential hazard to site workers, neighborhood public, downstream users, and the natural ecological community is needed. First, the Hazard Ranking System (Federal Register, 47 FR 10972, March 12, 1982) should be applied (e.g., Caldwell *et al.*, 1981) and then, if the potential hazard is high enough, a rapid physical-chemical screening approach should be taken (e.g. Turpin *et al.*, 1981). Other data would be obtained as discussed in the previous section (Mathis, 1981). The bioassessment protocol would be used to provide an initial assessment of acute toxicity and to prioritize the site relative to other sites.

- The site is characterized but the extent of contamination is unknown. In this case the waste site is not well defined and it is probable that the bioassessment protocol would be used to screen for the extent of contamination and provide input to the experimental design for more detailed assessment.

- The site is characterized and cleanup or other remedial action is being taken. In this case bioassessment would provide an estimate of remaining hazard, monitoring of incipient hazard, and would help in establishing and monitoring the boundaries of a required containment zone. Criteria related to bioassessment results would be based on samples collected at the boundaries of the containment zone or, for groundwater, as appropriate to projecting the effects of flow beyond the containment zone boundaries.

The first and second scenarios lead to the site priority or screening line of the bioassessment protocol. These data are needed before proceeding to the more detailed assessment. The screening assessment has the intent of providing a rapid survey of potential problems. The third scenario leads to the detailed assessment. The key step is to develop a sampling design appropriate to the site and potential hazards of the site.

Samples are collected at essentially three stations for the preliminary assessment: The core station (most impacted station), the site containment boundary, and a reference station (off site). Appropriate surface or ground water and soil samples are collected at each site for bioassessment. Bioassessment results for the core and boundary stations are compared to the reference site. Three replicate samples, randomly collected at each site, are composited for bioassessment.

Detailed assessment consists of transects and multiple stations designed to evaluate questions concerning the spread of toxic materials and the relative hazard associated with the samples. Generally, the transect would begin at the core station. Multiple stations along the containment boundary would be required. Optimum allocation of manpower and laboratory resources must be based on the Experimental Design and related concepts as discussed under that heading.

Response levels for each organism: high, intermediate, low or none, can be obtained for each sample. Points can be assigned for the response levels, summed, and priorities assigned according to relative biological hazard. Then, if appropriate, the sites can be further ranked according to other risk criteria having to do with fate and human effects considerations.

BIOLOGICAL TESTS FOR THE BIOASSESSMENT PROTOCOL

Two types of samples are tested, water and soil. Water samples are collected from surface and ground water sites or obtained as extracts from soil samples. Soil samples are collected from appropriate sites using grab sample apparatus. Soil core samples are not required but may be used. Descriptive data, methods of collection, and sample treatment and storage requirements for water and soil samples are described in the sampling chapter.

Organisms for use with water samples include algae, daphnids, and fish. Organisms for use with soil samples include decomposers, plant seeds and earthworms. These tests are summarized in Table 4, along with the target variables, appropriate sample type, and the response levels. These response levels are provisional at this time and provide guidance on the relative hazard associated with a given sample. The response levels will be refined as more data are obtained. Results are reported as actual measured EC_{50} or LC_{50} and then the samples are categorized into the appropriate response

Table 4

DEFINITION OF TOXICITY CATEGORIES FOR AQUATIC AND TERRESTRIAL ECOLOGICAL ASSAYS

Assay	Activity Measured	Sample Type[a]	MAD[b]	Units	Response Levels for LC_{50} or EC_{50} Concentrations[c]		
					High	Moderate	Low or Not Detectable
Freshwater Fish	96-hr LC_{50} (lethality)	S L	1 100	g/L percent	<0.01 <20	0.01-0.1 20-75	0.1-1 75-100
Freshwater Invertebrate	48-hr EC_{50} (immobilization)	S L	1 100	g/L percent	<0.01 <20	0.01-0.1 20-75	0.1-1 75-100
Freshwater Algae	96-hr EC_{50} (growth inhibition)	S L	1 100	g/L percent	<0.01 <20	0.1-0.1 20-75	0.1-1 75-100
Seed Germination and Root Elongation	115-hr EC_{50} (inhibit root elongation)	L	100	percent	<20	20-75	75-100
Earthworm Test	336-hr LC_{50}	S	500	g/kg	<50	50-500	500
Soil Respiration Test	336-hr EC_{50}	S L	500 100	g/kg percent	<50 <20	50-500 20-75	500 75-100

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[a] S = solid, L = aqueous liquid, includes water samples and elutiate or leachate. Nonaqueous liquids are evaluated on an individual basis due to variations in samples such as vehicle, percent organic vehicle, and percent solids.

[b] MAD = Maximum applicable dose.

[c] LC_{50} = Calculated concentration expected to kill 50 percent of population within the specified time interval.

EC_{50} = Calculated concentration expected to produce effect in 50 percent of population within the specified time interval.

level category.

The tests included in the bioassessment protocol were selected based on the validity of tests and their feasibility for use. Validity factors are the accuracy, precision, and sensitivity (precision at low concentration) of a test. Accuracy concerns how well the results can be extrapolated to a natural system. Precision and sensitivity are statistical factors and vary with the organisms and the type(s) of toxicants. Feasibility factors concern the cost of the test, the degree of expertise needed by the operator, the convenience of performing the test, and the speed with which a result is obtained. Although these data are available for some of the tests, ongoing research will provide a more complete compilation of these data. This information will be necessary in order to design sampling programs with the optimal allocation of sampling and testing resources.

POTENTIAL BIOASSESSMENT METHODS

A matrix of general organism types and major physiological processes versus soil and water is shown in Figure 3. The protocol methods cover a wide spectrum of types and processes but there are obvious gaps. An important gap, which is not shown, concerns bioaccumulation. Since bioaccumulation requires a long-term exposure, it is not included in the protocol. A possible research effort on octanol:water coefficients related to microbial responses, or direct microbial bioaccumulation tests could be useful for assessing bioaccumulation.

Organism Type	Physiological Process	Laboratory Biological Test[a]	
		Soil	Water[b]
Plant	Photosynthesis	-	-
	Growth	Germination, Root Elongation	Selenastrum
	Genetic	(Tradescantia)	-
Invertebrate	Respiration	-	-
	Lethality	Earthworm	Daphnids
	Growth	(Earthworm)	-
	Genetic	-	-
Vertebrate	Respiration	-	-
	Lethality	-	Fathead Minnow
	Growth	-	-
	Genetic	-	-
Decomposer	Enzyme	-	(Microtox)
	Growth	Litter Decomposition	-
	Genetic	(Ames Test)	(Ames Test)

[a] Parentheses indicate test needs more study before broad use; dash indicates no candidate test; otherwise the tests are in the protocol, Appendix A.

[b] Water includes surface and ground water samples or soil leachates.

Figure 3. Matrix of Tests as Related to Types of Organisms and Physiological Processes.

Although some specific processes, photosynthesis and respiration, are not included in the protocol, it would be feasible to develop rapid tests for evaluating them. Other gaps such as a vertebrate soil test should be disregarded because the benefits are small compared to the costs of developing and performing such tests. Existing tests such as mutagenetic response of the plant *Tradescantia*, the Ames test, and Microtox (Patent Pending) may have application as part of a protocol but more development of these techniques is required. Microbial enzyme tests exist that are rapid, inexpensive, and may provide ecologically accurate and meaningful results. Adaptation of these tests to evaluate hazardous waste sites could be a cost-effective study goal.

EXPERIMENTAL DESIGN

SCOPE

The sampling program must be designed so that answers to the two issues--site prioritization and cleanup evaluation--are obtained. These issues can be phrased as questions: In terms of relative toxicity how does the site rank compared to other sites in the state (or other defined region)? Are levels of toxic materials in soil or water samples sufficiently low that potential environmental hazard beyond the site boundary is minimal?

To begin answering these questions, risk, statistical design, sampling constraints, and the characteristics of the tests themselves must be considered. Each of these topics will be briefly discussed before providing a step-by-step sampling protocol.

RISK ASSESSMENT

Some element of risk assessment is involved in all decision making. To evaluate the potential risk to ecosystems from hazardous waste sites, a basic understanding of the concepts of risk assessment can help in defining the issues and significance of the method. In this section, the major concepts of risk assessment are discussed in general and then addressed specifically to the bioassessment protocol.

There are three broad steps in making a risk assessment: analyzing the system, determining the "dose-response" relation, and integrating these factors to estimate the risk. Note that defining the level of acceptable risk is essentially a political decision. Acceptable risk may be more a question of perception. Thus, decisions may be left either to the individual, for example, cigarette smoking, or to society, for example, nuclear radiation.

In analyzing the hazardous waste system, the composition and quantity of material released to the environment need to be estimated. Then, transport and migration should be defined for materials including any environmental transformations of compounds that would occur under the specific conditions at the site.

The dose-response relationship refers to the concept of relating some response to the concentration of material and, in most cases, the duration of exposure. A target variable must first be defined that bears a functional relationship to exposure. This is a key step since knowing the amount of material that is present is not meaningful without knowing that there is an effect, that a quantitative relation exists between concentration and effect, and that the effect is important ecologically and on a significant scale. The biological tests play an important role in developing these relationships.

After the above two steps are accomplished within reasonable confidence limits, the risk can be assessed by determining the quantity of material, the exposure to key parts of the system, and estimating the risk using the concentration-duration-effect relationship. This integration step requires many assumptions which must be clearly identified. For example, extension of results to low doses is difficult, inaccurate, and for statistical reasons, most costly to obtain. Thus, extrapolation is often practiced, and it may be based on assumptions that are incorrect.

The bioassessment protocol as it presently exists in this report does not deal explicitly with specific hazardous materials, their quantity, fate, or transformation. Instead, the biological tests measure responses to the mixture of materials obtained in a sample. The responses are directed at the concentrations available to organisms and at the mixture which may be different than the sum of the individual effects because of interactions. By analyzing transect samples, a response gradient can be determined that could be related to transport and migration processes.

The biological tests were developed using a concentration-duration-effect relationship (for example, Figure 4). Initially, this relationship was developed using one toxicant at a time. However, studies using multiple pollutants and complex waste mixtures as in Figure 4 have shown the concentration-duration-effect concept to exist. Thus, the concept of using dilutions of a complex waste in a synthetic or natural receiving medium appears valid.

There is one other issue of risk that must be considered. What are the effects of error? Two kinds of error exist; saying that there is a certain level of risk when in fact there is none and saying that there is none, when in fact a risk exists. In the first case, significant unnecessary expenditure might occur. In the second, considerable environmental damage might occur. It is important to evaluate both errors in a cost-benefit

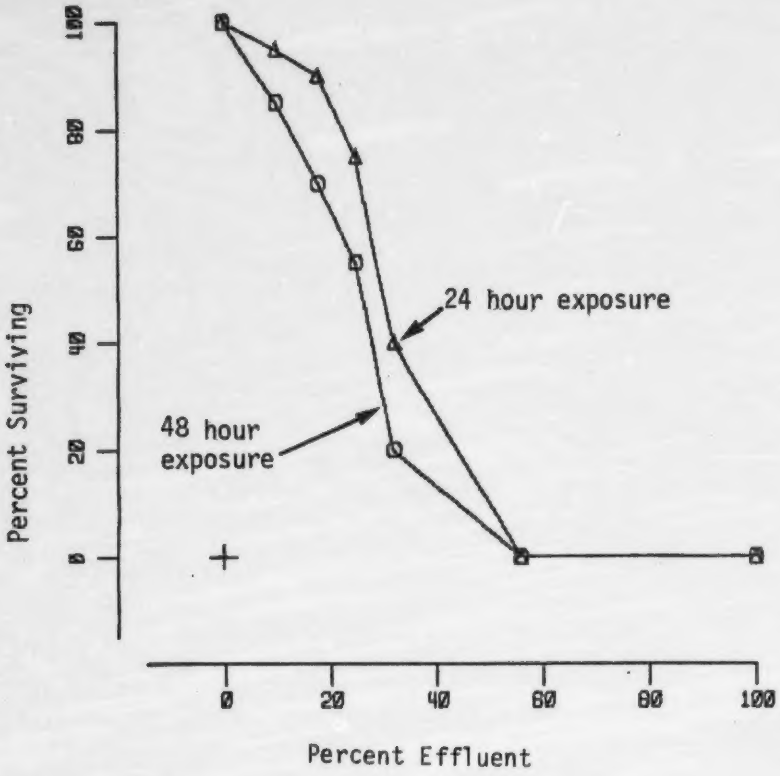

Figure 4. Concentration-duration Results From an Acute Toxicity Test Exposing Mysid Shrimp to a Simulated Refinery Effluent (data from Buikema, _et al_., 1982)

manner before defining acceptable risk. For example, if there is significant additional cleanup cost with little environmental benefit, it may be possible to accept a higher level of test response at the containment boundary. Conversely, the no-effect risk level might be appropriate for a cleanup cost associated with high hazard. These questions must be explored in more detail at specific sites.

The variance in the biological test results needs to be known as well as the variance in sampling. This information is used to allocate sampling and test resources. Without proper design, money is wasted and proper results may not be obtained. These factors are discussed qualitatively in the next section.

Some general approaches to risk analysis that provide further information on risk assessment have recently been discussed (Ricci and Molton, 1981; Starr and Whipple, 1980; Squire, 1981). A detailed review of the literature on carcinogenic risk assessment is contained in Krewski and Brown (1981) which has general applicability in the context of this report.

STATISTICAL CONSIDERATIONS AND EXPERIMENTAL DESIGN

The goal of using bioassessment procedures as a tool for the evaluation of the potential environmental hazards among existing sites is to detect differences within and among sites and to distinguish among groups of sites on the basis of this potential. To achieve these objectives, appropriate test procedures must be identified and experiments must be designed in such a manner that differences in the test results, which reflect the potential hazards, will be identified. Three main categories of statistical considerations which relate to these requirements are discussed herein. These are: the location of sample collection sites, experimental procedure specifications, and analytical techniques. Although discussed separately, these topics are not independent and decisions concerning each of these aspects affect the options available in the other levels of the sampling design.

The selection of sampling locations within the individual hazardous waste sites will be a function of the type of biological test to be performed, as well as uniformity of the site with respect to edaphic characteristics and habitat type. It is known that the relationship between hazardous waste concentrations and morphological, chemical and biological sampling area determinants will vary widely within the sampling sites. Yet at the same time, it is essential to the overall study design for the comparison among sites, that these variations be kept at a minimum. In this manner the effect of the individual hazardous waste sites is isolated and can be identified.

The feasibility of two procedures which will minimize the influence of within site variability on the estimated site effect should be evaluated. The first is the characterization of sample site-sample type relationships. The ability to specify sampling site selection criteria based on morphological, edaphic or biological characteristics for each proposed type of biological sample should be addressed. Certainly, tradeoffs must be made in the process of the specification of sampling area characteristics. This is because the very criteria which narrow the possibilities of sampling locations within waste sites can, at the same time, restrict the number of wastes sites throughout the United States where the biological test can be applied. However, for the purpose of reducing the sample variance within sites and equilibrating sample variances among sites, site-type characterization must be discussed.

Secondly, the feasibility of random sampling within the acceptable sampling area should be addressed. Random sampling is essential to the assumptions of many appropriate analytical techniques and, as discussed below, the larger the number of replicates the greater is the ability to distinguish among differences in the target variable or variables among sampling (hazardous waste) sites. In the overall study design, a stratified random sampling plan should be adopted with the strata identified on the basis of the set of descriptive criteria discussed above and random sampling within the strata.

Related to the question of the selection of sample location within individual waste sites is the specification of control samples. Each bioassessment procedure selected should be conducted with control or reference stations at each site. These samples will provide the means to assess the effects of the hazardous waste within the sites and will serve as quality control checks for the bioassessment program. Procedures for control samples (experiments) should be designed such that they can be repeated in the same manner at each site.

The second major area of statistical considerations which must be addressed is the appropriate level of sampling effort. Decisions on the level of sample replication or sampling effort in general cannot be made independently of careful consideration of the minimum level of difference in selected biological parameters that it is desired to detect and the precision with which differences should be detected. It is crucial to carefully plan field experiments in order to define these levels, to establish the number of samples required, and to specify the appropriate analytical approach.

Taking the analysis of variance (ANOVA) technique as an example of the type of statistical technique that might be used to identify differences in the mean values of specified measurements among the waste sites, sampling specifications and their implications to the overall study objectives will be discussed in the following paragraphs.

In considering the use of the ANOVA model, two significant criteria are specified. These are the probability of rejecting the null hypothesis when it is true (alpha probability, or Type I error) and the probability of accepting a null hypothesis when it is false (beta probability, or Type II error). Respectively, these errors are the probability of concluding there is a risk when no risk exists (Type I) and no risk when a risk does exist (Type II).

Results of statistical tests are often summarized by stating that no significant difference among stations was found at the 0.05 significance level. This refers to the alpha probability criterion which embodies the risk of mistakenly rejecting a null hypothesis that no differences exist. At a particular site having low potential for harm, one might select a less restrictive alpha criterion to minimize costs. The significance level of the beta parameter should also be specified, especially in the case of comparisons to determine the relative environmental risk from hazardous waste sites, because decision makers are also interested in the probability that the test was unable to detect a difference that did exist. Another statistical parameter, referred to as the power of a test (1-beta) is important in this context since it defines the probability of correctly detecting experimental effects (e.g. differences among waste sites) in a particular bioassessment procedure.

Closer examination of the beta probability and its complement, the power of a test, is instructive since these probabilities can be defined as a function of sample size. In this manner, the probability of the level of difference that can be reliably detected with alternative allocations of sampling resources (stations and replication) can be determined.

In addition to the value of such investigations in a posteriori analyses of results, the probability of detecting differences in the selected biological parameters between waste sites and the levels of differences which can be detected with proposed sampling designs should be determined, and this requires that the population variance of selected bioassessment parameters must be determined. This will be accomplished during the ongoing research being performed by the USEPA's Corvallis

Environmental Research Laboratory.

Besides the probability criteria, consideration must be given to the methods used to distinguish among waste sites or to categorize them according to enviromental risk. Quantitative methods are recommended, and a large array of parametric and nonparametric statistical techniques are available for this purpose. As indicated above in the discussion of sample replication, the allocation of sampling resources should be made so as to optimize the level of difference that can be reliably detected among sites.

Before the application of the parametric models, it must be determined if the underlying assumptions (i.e., homogeneity of variances, independence, and normality) can be met. In the case that the assumptions of parametric tests cannot be maintained by data transformations, nonparametric tests can be substituted. Although nonparametric tests lack the distributional assumptions of parametric tests, they have less power and they lack the ability to evaluate interactive effects.

The analysis of variance (ANOVA) is an example of a parametric statistical model which can be used to make univariate comparisons among waste-site samples. The purpose of the statistical model is to estimate true differences among the sample means. To describe possible effects of the waste site, a simple linear model is proposed by which any single observation can be decomposed as follows:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

where:

Y = biological observation for site i and replicate j

μ = mean value over all sites and relicates

α_i = effect of site i on Y

ε_{ij} = random deviation of the observation from its expectation $(\mu + \varepsilon_i)$.

The ANOVA is used to test the hypothesis that there is no difference in the biological observations made at different sites, i.e. that differences do not exist in the component due to site location $(\alpha_i = \alpha_2 = ... = \alpha_n)$.

In cases where significant differences are found to exist among sampling sites, multiple range tests (such as the Student-Neumann-Keuls or Duncan's) can be used to identify subsets of samples (sites) having equal mean values for the variable under examination. An example of the use of a multiple range test to demonstrate treatment differences among algal bioassays and to identify subsets having equivalent mean values is shown in Table 5.

In conclusion, decisions concerning the methods which will be used to distinguish among the waste sites sampled must be made in advance of the adoption of any bioassessment procedure. In this manner the bioassessment program can be optimized in order to provide the level of precision to assess potential hazards for the minimum costs.

Table 5

DUNCAN'S MULTIPLE RANGE TEST OF COMPLEX ADDITIONS TO SCENEDESMUS[a] (From Cleave, *et al.*, 1980)

max standing crop, X

treat. no.	concn, mL	treatment																					
1	20	AR elutriate																					*
25	20	BP elutriate																					*
27	10	BP elutriate																				*	
42		control																			*	*	
44		control																		*	*		
45		control																	*	*	*		
26	15	BP elutriate																	*	*	*		
21	20	BR salts																	*	*	*		
15	10	AP salts																*	*	*	*		
7	10	AR salts																*	*	*	*		
22	15	BR salts															*	*	*	*	*		
32	5	BP salts														*	*	*	*	*			
20	5	BR elutriate													*	*	*	*	*	*			
4	5	AR elutriate												*	*	*	*	*	*				
28	5	BP elutriate												*	*	*	*	*	*				
24	5	BR salts											*	*	*	*	*	*					
30	15	BP salts										*	*	*	*	*	*						
43		control									*	*	*	*	*	*							
33	20	AP column leachate								*	*	*	*	*	*	*							
10	15	AP elutriate								*	*	*	*	*	*	*							
6	15	AR salts								*	*	*	*	*	*	*							
9	20	AP elutriate							*	*	*	*	*	*	*								
23	10	BR salts							*	*	*	*	*	*	*								
31	10	BP salts							*	*	*	*	*	*	*								
3	10	AR elutriate							*	*	*	*	*	*									
39	10	AP column salts							*	*	*	*	*	*									
5	20	AR salts						*	*	*	*	*	*										
14	15	AP salts						*	*	*	*	*	*										
8	5	AR salts					*	*	*	*	*	*											
12	5	AP elutriate				*	*	*	*	*	*	*											
2	15	AR elutriate			*	*	*	*	*	*	*												
41		control		*	*	*	*	*	*	*													
16	5	AP salts		*	*	*	*	*	*	*													
38	15	AP column salts		*	*	*	*	*	*														
29	20	BP salts		*	*	*	*	*	*														
35	10	AP column leachate		*	*	*	*	*	*														
13	20	AP salts	*	*	*	*	*	*															
19	10	BR elutriate	*	*	*	*	*																
18	15	BR elutriate	*	*	*	*																	
37	20	AP column salts	*	*	*																		
36	5	AP column leachate	*	*																			
17	20	BR elutriate	*	*																			
34	15	AP column leachate	*																				
40	5	AP column salts	*																				
11	10	AP elutriate	*																				

max specific batch growth rate, $\hat{\mu}_b$

treat. no.	concn, mL	treatment																				
1	20	AR elutriate																				
41		control																				*
32	5	BP salts																			*	*
15	10	AP salts																			*	*
36	5	AP column leachate																		*	*	*
45		control																	*	*	*	*
44		control																	*	*	*	*
16	5	AP salts																	*	*	*	*
20	5	BR elutriate																	*	*	*	*
6	15	AR salts																*	*	*	*	*
31	10	BP salts															*	*	*	*	*	*
40	5	AP column salts														*	*	*	*	*	*	*
42		control														*	*	*	*	*	*	*
12	5	AP elutriate														*	*	*	*	*	*	*
21	20	BR salts													*	*	*	*	*	*	*	*
25	20	BP elutriate													*	*	*	*	*	*	*	*
13	20	AP salts												*	*	*	*	*	*	*	*	
30	15	BP salts											*	*	*	*	*	*	*	*	*	
34	15	AP column leachate										*	*	*	*	*	*	*	*	*	*	
43		control									*	*	*	*	*	*	*	*	*	*		
29	20	BP salts									*	*	*	*	*	*	*	*	*	*		
9	20	AP elutriate								*	*	*	*	*	*	*	*	*	*			
5	20	AR salts							*	*	*	*	*	*	*	*	*	*				
7	10	AR salts							*	*	*	*	*	*	*	*	*					
23	10	BR salts							*	*	*	*	*	*	*	*						
4	5	AR elutriate							*	*	*	*	*	*	*							
26	15	BP elutriate						*	*	*	*	*	*	*	*							
18	15	BR elutriate						*	*	*	*	*	*	*								
17	20	BR elutriate					*	*	*	*	*	*	*	*								
24	5	BR salts					*	*	*	*	*	*	*	*								
14	15	AP salts				*	*	*	*	*	*	*	*									
27	10	BP elutriate				*	*	*	*	*	*	*	*									
28	5	BP elutriate				*	*	*	*	*	*	*										
10	15	AP elutriate				*	*	*	*	*	*											
8	5	AR salts				*	*	*	*	*	*											
35	10	AP column leachate				*	*	*	*	*	*											
19	10	BR elutriate				*	*	*	*	*												
38	15	AP column salts				*	*	*	*													
33	20	AP column leachate				*	*	*	*													
39	10	AP column salts				*	*	*														
11	10	AP elutriate			*	*	*															
22	15	BR salts			*	*																
37	20	AP column salts			*																	
2	15	AR elutriate		*																		
3	10	AR elutriate	*																			

---

[a] Treatments are ranked from the lowest value at the top of the listing to the highest value at the bottom of the listing. Any groups of treatments that are not significantly different ($P < 0.05$) from each other are connected by a line of stars to the right of the ranking list.

GUIDELINES FOR SAMPLING

The primary reason for a sampling design is to allocate sampling and testing resources in an optimal manner. In the preliminary assessment, a minimal design of three replicate samples composited for analysis at each of three stations (core, boundary, reference stations) is specified, obviating the necessity for a statistical design. However, if resources permit and potential hazard is great enough, a statistical design should be used. For the detailed assessment, the number of stations and the number of sample replicates are defined during statistical design.

Three types of samples are obtained: soil samples, ground water, and surface water. A preliminary survey to observe physical-chemical-biological factors such as surface waters and flow directions, topography, types of habitats, site boundary conditions, aquifer locations and underground flow nets, and other ecological variables is an invaluable aid to selecting stations.

Each site will have specific characteristics that will cause a specific set of stations to be selected. The most samples and stations would arise from a situation where a groundwater aquifer underlies a disposal site, surface water is within the site containment boundary, and the area of direct disposal is large. Thus, the reference station, the core station, and the site boundary station would each require three types of samples: surface water, ground water, and soil. A total of 18 water samples and 9 soil samples would be obtained at that site using three replicate samples at each of the three stations. In addition, extracts from the soil would be tested. To minimize the number of tests, the samples would be composited for the biological testing. Such a hazardous waste site would have the experimental design shown in Table 6.

The final selection of sampling stations depends on the user-defined heirarchy of needed information related to the available resources. The methods for selecting stations and criteria for defining the number of replicates are based on the statistical analysis to be performed. Selected Type I error (alpha) levels and the desired power of the test (1-beta) then fix the number of stations and replicates required. Needed resources should be based on potential risk not on a budget figure. For example, if potential risk to ecosystems or society is relatively large or the cost of cleanup is relatively large, greater testing resources should be made available to insure that cleanup will be effective.

Preliminary Assessment

The preliminary assessment is used for initial prioritization and to determine the range of probable responses to be obtained with the biological tests. A minimum program consisting of the reference station, core (most impacted) station, and the site containment boundary station should be sampled. At least 3 replicate randomly selected samples should be obtained at each station and composited for testing.

If the biological tests uniformly give low or nondetectable response levels (Table 4), it is assumed that the site will be relatively risk free. Any test that shows a response at the intermediate or high level for a particular sample is cause for review and, probably, further analysis.

Table 6

MINIMAL EXPERIMENTAL DESIGN SHOWING THE MAXIMUM NUMBER OF SAMPLES (9) AND TESTS (36) FOR PRELIMINARY ASSESSMENT OF A SITE

	Tests and Samples to be Collected			
	Water Samples		Soil	
Station	Surface	Ground	Soil	Leachates[a]
Reference	Alga Daphnid Fish 1 sample[b]	Alga Daphnid Fish 1 sample[b]	Seeds Worms Microbes 1 sample[b]	Alga Daphnid Fish -
Core	Alga Daphnid Fish 1 sample[b]	Alga Daphnid Fish 1 sample[b]	Seeds Worms Microbes 1 sample[b]	Alga Daphnid Fish -
Boundary	Alga Daphnid Fish 1 sample[b]	Alga Daphnid Fish 1 sample[b]	Seeds Worms Microbes 1 sample[b]	Alga Daphnid Fish -

---

[a] Additional samples would not be collected because extracts would be obtained from soil samples.

[b] Composite sample from three random grab samples obtained from each station.

Then, the appropriate steps and decision points shown in Figure 2 would be followed.

It may be desirable to perform a statistically based sampling program (e.g., ANOVA) using the uncomposited samples to analyze the data. The variance of each biological test should be known. Expected variance in a particular biological test might be relatively high (coefficient of variation = 50 percent). Specifying appropriate levels of alpha (0.05 to 0.2) and the power of the test (1-beta = 0.8), one might wish to determine whether a difference could be shown at the intermediate response level (Table 4) for the site containment boundary sample compared to a reference station.

Detailed Assessment

Statistical methods should be used to evaluate the differences in the results of the bioassessment tests among the sampling stations. The analytical procedures must be specified in advance so that adequate sampling is conducted. Guidelines for selecting sampling locations within the individual sites should be developed. For example, attempts should be made to characterize optimal locations for individual sample types. In this manner sample variances will be reduced. Transects along major exposure routes or along site containment boundaries could be used.

A pilot or feasibility study for the purpose of determining the number and types of samples required and optimum allocation of resources should be performed. The preliminary assessment might serve for this purpose.

The error levels would be more conservative than those described for the preliminary assessment. For example, based on potential risk the following level could be selected, alpha = 0.1. Then the sampling stations and number of replicates would be specified according to resources needed to characterize the site and the expected results from the biological tests. The power of the test (1-beta) should be estimated and reported.

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APPENDIX A

BIOLOGICAL TESTS FOR
BIOASSESSMENT OF HAZARDOUS
WASTE SITES

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OVERVIEW OF
BIOASSESSMENT PROTOCOL

TEST PROCEDURES

A set of aquatic and terrestrial biological tests have been compiled to aid in assessing potential environmental hazard of hazardous waste sites. Users would select all tests or those appropriate to their needs and use them according to the procedures contained in this report.

The three tests directed at aquatic ecosystems are the algal bioassay, and the fish and *Daphnia* toxicity tests. The three terrestrial tests are the root elongation test, the earthworm acute toxicity test, and the soil litter microorganism test.

The aquatic tests are applied as appropriate to surface or ground water samples, extracts of soil samples, and nonaqueous samples where appropriate. The terrestrial tests are applied to soil extracts and soil samples.

Two levels of testing are defined, range finding and definitive, but more detailed sampling and testing designs can be devised for specific needs. Range finding tests are a geometric series of dilutions by factors of 10, for example, 1.0, 0.1, 0.01, 0.001. At least three dilutions should be performed. Definitive tests are a geometric series of dilution by factors of 2, for example, 1., 0.5, 0.25, 0.125, 0.0625, 0.03125. At least six dilutions should be performed. For the definitive test, concentrations usually bracket the intermediate value of the range-finding test or utilize the most effective value as the highest concentration. Soil or water are diluted in synthetic media (algal assay medium (AAM), reconstituted water, artificial soil) and, if appropriate, in unimpacted natural waters or soils from the site environs.

Control biological tests should always be performed. These include, where appropriate, negative, positive, solvent and reference controls (defined in the following section). The tests have been designed to be as reproducible as possible using carefully standardized test media and organisms. Quality assurance should follow the "Guidelines and Specifications for Implementing Quality Assurance Requirements" (USEPA, 1980). Safety procedures should be practiced to prevent exposure to staff (Appendix B).

The tests described in this appendix were taken from several important sources and readers should be aware of the references. The three aquatic tests and the root elongation test are from Brusick and Young, 1982, and the earthworm test is in development (contact C. Callahan, Corvallis ERL). The soil respiration test was largely provided by Lighthart and Bond (1976). Other major sources include the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975) and ASTM (1980), USEPA Methods (1979), Standard Methods (APHA, 1981), Miller _et_ _al_. (1978), and a review of statistical methods by Stephan (1977).

GLOSSARY

To insure a common understanding of terms in this report, a glossary has been included.

Response Variables

Effective Concentration (EC) - concentration that produces the desired effect at a specified level in a percent of the exposed population within a specified time.

96 hour EC_{50} - concentration that produces the desired effect in 50 percent of the population within 96 hours of exposure. Typical levels are 10, 20, 50, 100 percent.

Lethal Concentration (LC) - concentration that produces death as the effect at a specified level in a specified time.

Stimulating Concentration (SC) - concentration that causes growth to increase at a specified level in a specified time.

Controls

Negative Control - a test in which no toxicant is added to 100 percent dilution medium.

Positive Control - a test in which an effective concentration of a known toxicant is added to 100 percent dilution medium.

Solvent Control - a test in which the solvent used to extract the toxicant from a water or soil sample is evaluated. Then the solvent is added to 100 percent dilution medium at the same concentration as would occur with the extract.

Reference Controls - tests using natural water or soil samples collected from unimpacted areas of the site environs.

Sample Descriptions

Aqueous Sample - a receiving water (surface or ground water) or a soil extract obtained by extracting with water.

Non-Aqueous Sample - a water sample with more than 0.1 percent organic material (>1000 ppm), or any soil extract obtained by extraction with organic solvents.

Solid Sample - any solid phase material; generally, a soil sample.

Leachate - a sample of water that has percolated through a column of soil or other material such as waste.

Elutriate (extract) - a sample of water obtained by mixing a solid sample with a specified weight ratio of solvent, usually water, for a specified time and then separating from the solid phase by centrifugation and/or filtration.

Dilutions of Samples - a solid, aqueous or non-aqueous sample which is mixed homogeneously with natural soils or waters (receiving system) or standard soils or waters (artificial or reconstituted, reference or benchmark).

Standard Sample - any sample of constant or defined composition, e.g., synthetic water samples, soil conservation service regional soil sample, artificial soil.

Test Sample - site samples purposely contaminated with a known toxicant substance or mixture.

Contaminated Sample - site sample contaminated during site operation. Usually collected at boundary, or along a gradient.

Test Descriptions

Definitive Test - test used to establish the effective concentration of a substance or material. As used herein, the concentrations are in a geometric series with a ratio of 2.0.

Range-Finding Test - test used to determine the appropriate range of concentrations in which to apply the definitive test. The range-finding test can be entirely different from the definitive test, a slight modification of the definitive test, or an application of the definitive test over a broader range of concentrations. Generally, as used herein, the concentrations are in a geometric series with a ratio of 10.0.

Bioassessment Protocol - a combination of bioassessment tests for assessing potential environmental hazards at a site.

Bioassessment Procedure - a bioassessment test applied to a sample, for example, methods of evaluating algal growth in a soil extract.

Bioassessment Test - a specific biological population for assessing a biological response to a mixture of toxicants or a single toxicant. For example, the canary in the coal mine and the fish toxicity tests are Bioassessment Tests.

Bioaccumulation - uptake and retention of environmental substances by an organism from all sources (Veith *et al*., 1979).

Bioconcentration - uptake and retention of environmental substances by an organism from water (Veith *et al*., 1979). A bioconcentration factor (BCF) can be calculated as the quotient of the concentration of chemical in the tissue (or whole) of an aquatic organism divided by the concentration in the water in which the organism resides.

TEST SAMPLES

Tests are conducted on water, soil extracts or extracts, and soil samples collected as appropriate to the experimental design from the hazardous waste site. Dilutions are made into standard or reference water and soil samples. Sampling design should follow guidelines discussed in the text. In all cases, including actual sampling, transportation of samples, storage, pretreatment, dilution, and actual testing, procedures designed to provide protection of personnel safety and safety of the general public and of the environment must be carefully followed. Safety guidelines are discussed in Appendix B.

REPORTING

The test record should include where applicable:

- name and address of test laboratory
- date or period of testing
- name of person responsible for testing
- number of tests carried out (rangefinding and definitive tests)
- exact description of test conditions

- details of any variation of test materials and conditions from protocol
- details of test organism (age, maintenance and breeding conditions, source of supply)
- average live weight and range and number of organisms per dose at start and end of test
- description of obvious physical or pathological symptoms or distinct changes in behavior observed in test results
- graph showing concentration/effect curve
- mortality and changes in weight for control animals
- where appropriate, mortality and changes in weight for animals used as control, reference, or test animals
- date and signature of the person performing the test
- any other documents on test conditions

RESPONSE LEVELS

Generalized response levels for the 3 aquatic and 3 terrestrial tests are summarized in Table A-1. The qualitatively defined levels are presented as guidance. They are intended to help users to evaluate relative toxicity of specific samples. However, the response levels are not fixed values and further results will be incorporated to obtain better qualitative estimates of toxicity. Furthermore, the low or not detectable levels may be misleading since a lack of strong toxicity does not necessarily mean a sample is "safe"; it only is an indication of the immediate potential hazard due to toxicity.

Table A-1

DEFINITION OF TOXICITY CATEGORIES FOR AQUATIC AND TERRESTRIAL ECOLOGICAL ASSAYS

Assay	Activity Measured	Sample Type[a]	MAD[b]	Units	Response Levels for LC_{50} or EC_{50} Concentrations[c]		
					High	Moderate	Low or Not Detectable
Freshwater Fish	96-hr LC_{50} (lethality)	S L	1 100	g/L percent	<0.01 <20	0.01-0.1 20-75	0.1-1 75-100
Freshwater Invertebrate	48-hr EC_{50} (immobilization)	S L	1 100	g/L percent	<0.01 <20	0.01-0.1 20-75	0.1-1 75-100
Freshwater Algae	96-hr EC_{50} (growth inhibition)	S L	1 100	g/L percent	<0.01 <20	0.1-0.1 20-75	0.1-1 75-100
Seed Germination and Root Elongation	115-hr EC_{50} (inhibit root elongation)	L	100	percent	<20	20-75	75-100
Earthworm Test	336-hr LC_{50}	S	500	g/kg	<50	50-500	500
Soil Respiration Test	336-hr EC_{50}	S L	500 100	g/kg percent	<50 <20	50-500 20-75	500 75-100

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[a] S = solid, L = aqueous liquid, includes water samples and elutiate or leachate. Nonaqueous liquids are evaluated on an individual basis due to variations in samples such as vehicle, percent organic vehicle, and percent solids.

[b] MAD = Maximum applicable dose.

[c] LC_{50} = Calculated concentration expected to kill 50 percent of population within the specified time interval.

EC_{50} = Calculated concentration expected to produce effect in 50 percent of population within the specified time interval.

GENERAL MATERIALS AND METHODS FOR BIOLOGICAL TESTS OF WATER SAMPLES

GENERAL INSTRUCTIONS

Setup and Preparation

The recommended test organisms in freshwater tests are the algae, *Selenastrum capricornutum*, the juvenile fathead minnow, *Pimephales promelas*, and early instars of *Daphnia magna*. The recommended test period is 96 hours for the algal test, 96 hours for the fish test, and 48 hours for the daphnid test. Thus, the principal finding obtained from an algal study is the 96-hour EC_{50}, EC_{90} or SC_{20}, from the fish study the 96-hour LC_{50}, and from the daphnid study the 48-hour EC_{50}.

The procedures for the fresh water tests have been developed largely from previous work (Brusick and Young, 1982). Modifications to the original protocols have been made where necessary to adapt tests to the requirements of the Bioassessment Protocol.

Materials and methods that are common to all, or nearly all, aquatic tests are presented in this section. The section for each specific test discusses materials and methods unique for that test and identifies which of the general materials and methods are applicable.

Facilities

The facilities should include tanks equipped for temperature control and aeration for holding and acclimating test organisms, and a constant temperature area or recirculating water bath for the test vessels. If the use of reconstituted dilution water is necessary, there should be a tank for its preparation. If air is used for aeration, it must be free of oil and fumes. The test facility must be well ventilated and free of fumes. Illumination should be provided of an intensity and duration that is specified in the Materials and Methods section for each test.

Construction Materials

Materials that come in contact with samples, stock solutions, or test solutions should minimize sorption of any constituents of the test material and not contain any substances that can be leached or dissolved by the water. Glass, #316 stainless steel, and perfluorocarbon plastics must be used whenever possible to minimize leaching, dissolution, and sorption. Unplasticized plastics may be used for holding and acclimation tanks and in the water supply system. Rubber, copper, brass, galvanized, and lead must be avoided. If stainless steel is used it must be welded, never soldered. Silicone adhesive used to cement glass containers sorbs some organochlorine and organophosphorus compounds which are difficult to remove; therefore, as little adhesive as possible should be in contact with test material solutions and extra beads of adhesive should be on the outside, not the inside, of the containers.

Test Containers

Fish tests should be conducted in 20-liter wide-mouth soft-glass jars or in all-glass containers 30 cm wide, 60 cm long and 30 cm high. Daphnids should be exposed in 4-liter wide-mouth soft-glass bottles, in 3.3-liter battery jars or in 250-milliliter beakers. Algal tests should be conducted in Erlenmeyer culture flasks of Pyrex or Kimax type of glass. The flask size is not critical, but due to CO_2 limitations the volume-to-volume ratio is. The recommended contents-to-flask-volume ratios for hand shaken flasks are:

25 ml in 125 ml flask
50 ml in 250 ml flask
100 ml in 500 ml flask

Maximum permissible contents-to-volume ratios in continously shaken flasks should not exceed 50 percent.

Cleaning and Preparation of Glassware

Each testing container must be cleaned before use. A new container must be (1) washed with non-phosphate detergent, (2) rinsed with 100 percent acetone, (3) rinsed with water, (4) rinsed with 10 percent nitric acid, (5) rinsed thoroughly with tap or other clean water, and (6) a final rinse with distilled or deionized water (3 volumes). After testing, each container should be cleaned as above unless the container is discarded.

For fish bioassays, disinfect test containers for 1 hour with an iodophor, 200 mg hypochlorite per liter, or a quaternary ammonium salt such as 800 ppm Roccal II (National Laboratories, Montvale, New Jersey 07645) with at least one thorough scrubbing during the hour, then rinse thoroughly. For safety, do not use acid and hypochlorite together.

All glassware used in algal testing is prepared as above. Flasks are dried in an oven at 50^{o} to $70^{o}C$. Demonstrably nontoxic plugs (for example, Gaymar white, polyurethane or equivalent, Gaymar Industries, Orchard Park, New York 14127) are inserted and the glassware is autoclaved for 20 minutes at 1.1 kg/cm^2 (15 psi) and $121^{o}C$. Cooled flasks are stored in closed cabinets.

Receipt and Quarantine for Fish

Stock fish shipped from outside sources may have been subjected to changes in temperature, dissolved oxygen and pH, handling disturbances, and other stresses, and should be examined carefully for health and vigor. Introduce holding water gradually into the shipping bags, observing the fish for abnormal behavior. When the difference in water temperature between the bag and holding tank is 2^{o} or less, fish from one bag should be introduced into the tank and observed for five minutes for acute stress. If acute stress is not seen, the remaining fish may be introduced into the tank in a similar manner.

To prevent spread of disease, incoming fish for stock should be quarantined for at least 2 weeks and observed for abnormal behavior and parasites. The quarantine tanks should be prepared in advance by thorough scrubbing and cleaning with an industrial cleaner, rinsing with water, sterilizing with a quaternary ammonium salt such as 800 ppm Roccal II, and rinsing with at least three changes of water before filling with dilution water. If after 2-weeks' quarantine they show no signs of infection or abnormal behavior they are transferred to stock holding tanks, otherwise, they are either discarded or treated as described in Disease Treatment for Fish, below.

To prevent initiation and spread of disease, nets, buckets, fish graders, and hands should be routinely disinfected with 200 ppm Roccal II before being placed in the water.

Disease Treatment for Fish

Freshwater fish may be chemically treated to cure or prevent diseases by using the treatments recommended in Table A-2. However, if a group of fish is severely diseased, the entire lot should be destroyed. Generally, the fish should not be treated during the first 16 hours after arrival at the facility because they may be stressed due to collection or transportation and some may have been treated just prior to transit. Tests must not begin with treated fish for at least 4 days after treatment. Tanks and test chambers which may be contaminated with undesirable microorganisms should be disinfected following the procedures outlined in Cleaning and Preparation of Glassware, above in this section.

Performing the Tests

Test Material

All samples and test materials must be handled according to safe procedures that protect the workers, society, and the ecosystem. These procedures are described in Appendix B. The test material may be a solid, aqueous liquid, or nonaqueous liquid. For the quantity of sample required to run each test, see Table A-3. Samples are usually tested directly without preparation, however, some test materials require pretest preparation. Except for the algal test, the aqueous sample (extract or water sample) should be run directly in the dilution water and must not be

Table A-2

RECOMMENDED PROPHYLACTIC AND THERAPEUTIC TREATMENTS FOR FRESHWATER FISH (from Brusick and Young, 1982)[a]

Disease	Chemical	Conc., mg/l	Application
External bacteria	Benzalkonium chloride (Hyamine 1622®)	1-2 AI[b]	30-60 min[c]
	Nitrofurazone (water mix)	3-5 AI	30-60 min[c]
	Neomycin sulfate	25	30-60 min[c]
	Oxytetracycline hydrochloride (water soluble)	25 AI	30-60 min[c]
Monogenetic trematodes fungi, and external protozoa[d]	Formalin plus zinc-free malachite green oxalate	25 0.1	1-2 hours[c]
	Formalin	150-250	30-60 min[c]
	Potassium permanganate	2-6	30-60 min[c]
	Sodium chloride	15,000-30,000	5-10 min dip
		2000-4000	[c,e]
	Dexon (35% AI)	20	30-60 min[c]
Parasitic copepods	Trichlorfon (Masolen®)	0.25 AI	[f]

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[a] These recommendations do not imply that these treatments have been cleared or registered for these uses. Appropriate State and Federal regulatory agencies should be consulted to determine if the treatment in question can be used and under what conditions the uses are permitted. These treatments should be used only on fish intended for research. They have been found dependable, but efficacy against diseases and toxicity to fish may be altered by temperature or water quality. Researchers are cautioned to test treatments on small lots of fish before making large-scale applications. Prevention of disease is preferred, and newly acquired fish should be treated with the formalin-malachite green combination on three alternate days if possible. However, in general, fish should not be treated on the first day they are in the facility. This table is merely an attempt to indicate the order or preference of treatments that have been reported to be effective. Before a treatment is used, additional information should be obtained from such sources as Davis (1953), Hoffman and Meyer (1974), Reichenback-Klinke and Elkan (1965) Snieszko (1970), and van Duijn (1973).

[b] AI - active ingredient.

[c] Treatment may be accomplished by (1) transferring the fish to a static treatment tank and back to a holding tank; (2) temporarily stopping the flow in a flow-through system, treating the fish in a static manner, and then resuming the flow to flush out the chemical, or (3) continuously adding a stock solution of the chemical to a flow-through system by means of a metered flow or the technique of Mount and Brungs (1967).

[d] One treatment is usually sufficient except for "Ich", which must be treated daily or every other day until no sign of the protozoan remains. This may take 4 to 5 weeks at 5 to 10°C and 11 to 13 days at 15 to 21°C. A temperature of 32°C is lethal to Ich in 1 week.

[e] Minimum of 24 hours, but may be continued indefinitely.

[f] Continuous treatment should be employed in static or flow-through systems until no copepods remain, except that treatment should not be continued for over 4 weeks and should not be used above 27°C.

Table A-3

SAMPLE SIZE REQUIREMENTS FOR AQUATIC ECOLOGICAL ASSAYS
(From Brusick and Young, 1982)

Type of Test	Solid (grams)	Liquid (liters) Aqueous	Nonaqueous[a]
Freshwater Fish	100 (75)[b]	100L (75L)	0.100 (0.075)
Freshwater Invertebrate	10 (4)	10L (4L)	0.010 (0.004)
Freshwater Algae	2 (1)	1L (0.06L)	0.010 (0.005)

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[a] Nonaqueous liquids include aqueous samples with greater than 0.2% organics, nonaqueous liquids, solvent exchange samples, and extracts or leachates in a nonaqueous (organic) vehicle.

[b] The first value given is the requested sample size for routing testing. The value in parentheses is the minimum feasible sample size to conduct the test.

aerated or altered in any way, except that it may be filtered through a sieve or screen with holes 2mm or larger to remove large particles. Aqueous samples should be filtered (0.45 micrometer cellulose acetate filters) to remove indigenous algae for the algal assay. This should be done as soon after collection as possible (on site is preferable) and must be done before sample storage. Solid and nonaqueous samples may be added directly by weight or volume respectively, diluted with dilution water and small subsamples of equal volume added to each test container. Samples must be covered at all times and violent agitation must be avoided. Undissolved materials must be uniformly dispersed by gentle agitation immediately before a portion of the sample is taken for use.

If testing is to be done on-site, the tests should begin within 8 hours of collection. If testing is to be done at a laboratory, the samples should be placed on ice for preservation during transportation. Testing should be performed as soon as possible after laboratory receipt of the samples. Samples should be stored at 4°C if testing is not initiated upon sample receipt. The temperature of the sample should be adjusted to that of the test (±2°C) before portions are added to the dilution water. Solid materials may be added directly to dilution water.

When diluting samples containing highly volatile substances, it may be desirable to add the test sample below the surface of the dilution water. Complete and accurate records of collection methods, treatments, and addition techniques must be maintained.

Sample Test Concentrations

Preparation of Toxicant. Depending on its nature, the test material is prepared by one of two methods. In the first method, solids or non-aqueous liquid materials may be added directly by weight or volume respectively to a stock solution or to the dilution water. The stock solution may be deionized water or a solvent and then equal volume subsamples of a small size are added to each treatment. If it is not possible to prepare a homogenous solution of the toxicant, it must be added directly to the dilution water in each replicate flask or tank.

The second method is for aqueous samples and allows testing by percent volume (volume/volume). Up to 100 percent of the sample with filter-sterilization if required, is used in the test. Additional test concentrations are prepared on a volume-percent basis by mixing appropriate volumes of sample with appropriate dilution water or medium.

Controls should consist of the dilution water or nutrient medium, and a receiving water sample if appropriate. These are called negative controls. It may be necessary to perform a range finding test with broad dilution limits (factors of 10, 100, 1000) before performing the final tests. Specific requirements are listed in the Section on Test Procedure for each test.

Dissolved Oxygen Concentration. Aeration of test solutions during the test should be avoided to minimize loss of highly volatile materials. Dissolved oxygen must be brought at least to minimum standards (40 percent of saturation) by dilution. If the dissolved oxygen concentration is less than 40 percent saturation in any test chamber for fish or *Daphnia* tests, this should be noted in the final report. Algal tests do not have defined dissolved oxygen concentration requirements.

FRESHWATER ALGAE 96-HOUR TEST

Introduction and Rationale

Unicellular algae are important producers of oxygen and form the basis of the food web in aquatic ecosystems. Since algal species and communities are sensitive to environmental changes, growth may be inhibited or stimulated by the presence of pollutants. Therefore, the response of algae must be considered when assessing the potential ecological effects of industrial or municipal discharges on aquatic ecosystems.

A simple screening test for toxicity to algae can be conducted in 96 hours. Algae are exposed to various concentrations of the test material and growth is measured at 96 hours. Results are expressed in terms of the EC_{90} (the lowest test concentration causing inhibition of growth by equal or greater than 90 percent relative to the control), and EC_{50} (the lowest test concentration causing inhibition of growth by equal or greater than 50 percent relative to the control). Stimulatory effects, if any, should be noted and expressed mathematically in terms of SC_{20} and used for estimation of bioactivity of the sample.

Materials and Methods

General procedures listed for all aquatic tests in the GENERAL INSTRUCTIONS are applicable to the static acute toxicity test with freshwater algae. Specific areas discussed in the GENERAL INSTRUCTIONS that should be followed are: facilities, construction materials, test containers, cleaning and preparation of glassware, and test material. Materials and methods unique to freshwater algal tests are included below.

Equipment

Equipment should include a constant-temperature room or incubator capable of providing temperature control of 24 $\pm 2^{\circ}C$. Daily hand shaking or a gyrotary shaking apparatus capable of 100 oscillations per minute should be used for test culture flasks. Continuous illumination of 4300 ±430 lumens/m^2 (400 ft-c) is required for freshwater green algae. Overhead cool-white fluorescent bulbs should be used. Light intensity is measured adjacent to the flask at liquid level using a light meter capable of being calibrated against National Bureau of Standards lamps. Culture containers for this and other aquatic tests are discussed in the Test Container section.

Freshwater Algal Nutrient Medium

Algal Assay Medium (AAM) is prepared by adding 1.0 ml of each of the macronutrient and micronutrient stock solutions, in the order listed in Table A-4, to 900-ml of filter-sterilized deionized water, with mixing after each addition. Then the final volume is brought to 1 liter with filter-sterilized deionized water. Deionized water is filter-sterilized by passing through a 0.45 micrometer porosity cellulose acetate membrane filter (pre-rinsed with 100-ml deionized water) into a sterile container. Medium should be constituted as needed but can be stored in the dark at 4°C to reduce possible photochemical changes and bacterial growth for periods up to one month.

Test Organisms and Culture Maintenance

For freshwater algal assays, the recommended test organism is *Selenastrum capricornutum*, a unicellular non-motile chlorophyte that is easily maintained in laboratory cultures. Obtain algal cultures (Culture No. ATCC 22662) from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852.

Upon receipt of the algal culture, approximately 1.0 ml should be aseptically transferred to the AAM. The rest of the culture can be maintained up to six months in a dark refrigerator at 4°C. Weekly aseptic routine stock transfer is recommended to maintain a continuous supply of "healthy" cells for experimental work. To retain a unialgal culture over a

Table A-4

COMPOSITION OF ALGAL ASSAY MEDIUM (AAM)

(From Miller *et al*., 1978)

Macronutrients

Stock Solutions[a]		Nutrient Composition Prepared Medium	
Compound	Concentration (g/l)	Element	Concentration (mg/l)
$NaNO_3$	25.500	N	4.200
$NaHCO_3$	15.000	Na	11.001
		C	2.143
K_2HPO_4	1.044	K	0.469
		P	0.186
$MgSO_4 \cdot 7H_2O$	14.700	S	1.911
$MgCl_2 \cdot 6H_2O$	12.164	Mg	2.904
$CaCl_2 \cdot 2H_2O$	4.410	Ca	1.202

Micronutrients

Stock Solutions[a]		Nutrient Composition Prepared Medium	
Compound	Concentration (mg/l)	Element	Concentration (ug/l)
H_3BO_3	185.520	B	32.460
$MnCl_2 \cdot 4H_2O$	415.610	Mn	115.374
$ZnCl_2$	3.271	Zn	1.570
$CoCl_2 \cdot 6H_2O$	1.428	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.250	Mo	2.878
$FeCl_3 \cdot 6H_2O$	160.000	Fe	33.051
$Na_2EDTA \cdot 2H_2O$	300.000	---	---

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[a] Other forms of the salts may be used as long as the resulting concentrations of elements are the same.

long period of time it is advantageous to prepare a semi-solid medium containing 1.0 percent agar made up with AAM, autoclaved and cooled to $45^{o}C$, and placed in sterile Petri plates. A portion of a liquid algal culture is streaked onto it and incubated under standard conditions. Algae should be transferred onto fresh plates every four weeks. Fresh liquid cultures should be started by transfer of a single algal colony to liquid medium at four week intervals. For test inoculation, liquid cultures are used.

A 6- to 8-day-old stock culture is used as the inoculum source. Population density in the stock culture is determined by direct counting or spectrophotometry with a standard curve. The culture should always be checked microscopically to insure that it is unialgal and healthy. A volume of inoculum calculated to provide a concentration of 10,000 cells/ml in the test concentration at the start of the test is aseptically added to each test flask. The volume of inoculum added should be between 0.1 and 1.0 ml. See the section on Response Monitoring to determine cell counts.

Test Procedure

Three replicates of each test concentration and control are tested. Prior to conducting the rangefinding or definitive 96 hr EC_{50} test, the 100 percent sample should be assayed with and without the addition of stock nutrient solutions equivalent to full-strength algal assay medium. If inhibition in the 100 percent sample plus AAM is less than 50 percent of the control, no further testing is necessary. Samples causing greater than 50 percent inhibition should be assayed by diluting the 100 percent sample (without nutrient addition) with algal assay medium to prepare each dilution series. This assures known nutrient availability in the test dilutions to calculate algal yield. Stock nutrient solutions are added to the 100 percent sample as in making up AAM to insure nutrient salts are equivalent to 100 percent AAM. Full strength AAM is used to dilute samples for the dilution series.

A range finding test will probably be necessary before running the actual test. A control plus concentrations of 80 percent, 10 percent, 1 percent and 0.1 percent (W/V or V/V) are usually necessary using 3 replicates each. The addition of AAM to make the 80 percent samples insures adequate nutrients for the test, while avoiding chemical effects or nutrient inavailability which occurs when using 100 percent AAM in test samples. The definitive test will span the moderate response concentration(s) using a geometric series. For example, if 1 percent (0.01) and 10 percent (0.10) gave toxic responses (EC_{50}), a test series would include: 0.1, 0.05, 0.025, 0,0125, 0.00625, 0.003125. solutions (W/V or V/V).

Controls include the AAM (negative control) to check standard organism response and receiving water if applicable (reference control), a solvent control if applicable (dilution water plus solvent). The positive control is applied with $ZnCl_2$ in AAM at a concentration of 80 ug Zn^{++}/l to give a range of inhibition of 51-66 percent (long term mean = 58.8).

Response Monitoring

After 96 hours of exposure, algal growth is measured by any of the following methods: (a) electronic particle counting, (b) biomass (dry weight), (c) absorbance, or (d) microscopic counts. Cursory microscopic observation is desirable to reveal and record any abnormalities in cell shape or condition. Because the algal test is designed to provide a comparative response to varying dilutions of sample dilute, it is better to use an electronic particle counter to measure growth. Other techniques can be used (Brusick and Young, 1982; Miller *et al*., 1978; American Public Health Association, 1981; Porcella and Cleave, 1981) but results must be reported along with conversion equations as mg/l dry weight.

Electronic Particle Counting

A Model ZBI Coulter Counter with Mean Cell Volume (MCV or MHR) Computer is used. The particle counter offers the greatest precision and accuracy and is the preferred method if equipment is available and samples are suitable. The MHR Computer must be calibrated with the organic calibration material; biomass may be determined indirectly by the following equation:

Cell counts (cells/ml) x MCV (m^3) x (2.9 X 10^{-7})* =
mg dry weight *S*. *capricornutum*/liter

*Note that the conversion factor of 2.9 x 10^{-7} may differ between laboratories and should be determined by each investigator. The standard reference particle (Part Number 1607081) can be obtained from Coulter Electronics, Inc., Hialeah, Florida.

If there are particles in the test material, it is possible to eliminate counts contributed by other particles. Uninoculated flasks are counted and these counts subtracted from the total counts. Then dry weights of cells can be calculated with the above formula. Particles may clog the aperture, and in such cases, another method should be used. The advantage of this method is that it allows for determination of biomass produced in addition to cell numbers.

Biomass (dry weight)

For this method, a measured portion of algal suspension is filtered through a tared 0.6 micrometer PVC membrane filter. The filters are prepared as follows: Dry for two hours at 70°C in an oven. Place filters in folded sheets of paper or aluminum weighting dishes on which the weights or codes are written. Cool in a desiccator for at lease one hour and weigh. Filter a suitable portion of culture (50 ml or less as the cell density dictates) under a vacuum of 51 kPa. Rinse filter funnel with 50 ml distilled water using a wash bottle and let the rinsings pass through the filter. Dry at 70°C, cool in desiccator, and weigh. Subtract tare weight, divide by volume (liters) of culture filtered and express as mg/l dry weight.

Absorbance

Measure absorbance with a spectrophotometer or colorimeter at a wavelength of 750 nm. Report instrument make and model, geometry and path length of the cuvette, wavelength used, and the equivalence to biomass (absorbance units per milligram dry weight per liter).

Limit photometric measurement of absorbance to a range of $0.05 \leq D \leq 1.0$, where D represents optical density. Samples can be diluted to obtain the appropriate range.

Microscopic Counting

To determine cell numbers in the inoculum or create a standard curve for later absorbance, direct microscopic counts are necessary. A hemacytometer counting chamber and a microscope are used. Two samples are taken from each flask, and two counts are made of each sample. Whenever feasible, 400 cells per replicate are counted in order to obtain ±10 percent accuracy at the 95 percent confidence level. Generally this method is the least feasible for monitoring test responses.

Results and Data Interpretation

Calculations. Percent inhibition (I), or stimulation (S), is calculated for each concentration according to the following formula:

$$\frac{(T\text{-}IN) - P(C\text{-}IN)}{P\,(C\text{-}IN)} \times 100 = \begin{matrix}(+)\% = \text{Stimulation} \\ (-)\% = \text{Inhibition}\end{matrix}$$

where

P = percent volume of AAM used to dilute the test sample; must not be less than 20 percent.

C = maximum standing crop (mg/l) obtained in the AAM control.

T = maximum standing crop (mg/l) obtained in the test sample.

IN = dry weight (mg/l) of inoculum used at start of test.

Four toxic concentrations must be tested in the definitive test. They are: the concentrations which inhibit less than or equal to 25 percent; from 25 percent up to 50 percent; from 50 percent up to 75 percent; and the lowest concentration which inhibits more than 75 but less than or equal to 90 percent of the test algae. Three endpoints may be calculated from the percent response vs. concentration data. For samples which are inhibitory, an EC_{90} (defined as the lowest test concentration causing growth inhibition of 90 percent relative to control) and an EC_{50} (defined as the lowest test concentration causing growth inhibition of 50 percent relative control) are calculated. For samples which are stimulatory, an SC_{20} (defined as the lowest concentration causing growth stimulation of 20 percent relative to control) is calculated. For all samples, the EC_{50}, EC_{90}, and SC_{20} are calculated using any of several statistical methods.

The 96-hour EC_{50} results are evaluated according to criteria defined in Table A-1 which will permit the test material to be ranked by toxicity category. While the EC_{90} endpoint may be the most meaningful biological effect for long-term impact on the environment, the more sensitive EC_{50} is used in this assay to rank samples.

STATIC ACUTE TOXICITY TESTS WITH FRESHWATER FISH AND DAPHNIA

Introduction and Rationale

The static toxicity tests with freshwater fish and Daphnia utilize juvenile fathead minnows, Pimephales promelas, and early instars of Daphnia magna. The static acute exposure period is 96 hours for the fathead minnow and 48 hours for the daphnid study. The 96-hour mean lethal concentration (96-hour LC_{50}) is calculated for the fathead minnow. Because death is not always easily determined in Daphnia, the 48-hour effective concentration (48-hour EC_{50}) is calculated for Daphnia.

Materials and Methods

Procedures listed for all aquatic tests under GENERAL INSTRUCTIONS are applicable to the static acute toxicity tests with freshwater fish and Daphnia. Sections that should be followed are: Facilities, Construction Materials, Test Containers, Cleaning and Preparation of Glassware, Receipt and Quarantine for Fish, Disease Treatment for Fish, Test Material, and Dissolved Oxygen Concentration. Materials and methods unique to freshwater fish and Daphnia tests are included below.

Dilution Water

Dilution water can be from the site (upstream of possible contamination) local dechlorinated tap water, or reconstituted water. A minimal criterion for an acceptable dilution water is that healthy organisms will survive in it for the duration of acclimation and testing without showing signs of stress such as discoloration or unusual behavior. Water in which daphnids survive and reproduce satisfactorily should be an acceptable dilution water for tests with freshwater organisms.

The dilution water should be of constant quality and should be analyzed by accepted methods (Durrant _et al._, 1974; Imai and Siegel, 1973; Santelman, 1972; Walley _et al._, 1974) to ascertain that none of the following substances exceeds the maximum allowable concentration shown:

Pollutants	Maximum Concentration
Suspended solids	20 mg/l
Total organic carbon	10 mg/l
Un-ionized ammonia	20 ug/l
Residual chlorine	3 ug/l
Total organophosphorus pesticides	50 ng/l
Total organochlorine pesticides plus PCB's	50 ng/l

The dilution water is considered to be of constant quality if the monthly ranges of the hardness, alkalinity, and conductivity are within 10 percent of their respective means and if the monthly range of pH is less than 0.4 units. Reconstituted dilution water may be prepared according to the method shown in Table A-5. For comparability of results between tests, the hardness should be as close as possible to 100 mg/l as $CaCO_3$.

Table A-5

RECOMMENDED COMPOSITION FOR RECONSTITUTED FRESH WATER THAT IS MODERATELY HARD (calculated from ASTM, 1980)

Salts Added to Distilled Water*, mg/l	
$CaSO_4 \cdot 2pH_2O$	70
$MgSO_4$	70
KCl	4.5
$NaHCO_3$	168

Water Quality	
H (air equilibrated)	8.3
Hardness, mg/l as $CaCO_3$	100
Alkalinity, mg/l as $CaCO_3$	100
Total dissolved solids	250

* Stock solutions of individual salts can be prepared so that 10 ml in one liter produces the desired final concentration. Store stock solutions in the dark at 4°C.

Species

The juvenile fathead minnow, Pimephales promelas, and early instars of Daphnia magna are the species to be used in Level 1 freshwater static acute toxicity tests. The fathead minnow is a warm-water fish of ponds, lakes, and sluggish streams. Daphnids occur in nearly all types of freshwater habitats. Both species, have been recommended as bioassay organisms by the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975; ASTM, 1980) because of their wide geographic distribution, important role in the aquatic food web, temperature requirements, wide pH tolerance, ready availability, and ease of culture.

Source

Fathead minnows may be obtained from private, state, or federal fish hatcheries, or captured from wild populations in relatively unpolluted areas. However, collecting permits may be required by local and state agencies. Fish collected by electroshocking should not be used. Daphnia should be reared in the testing facility from laboratory cultures.

Sizes, Life Stages

Fathead minnows used in testing should weigh between 0.5 and 1.0 g each. All fish in each test should be from the same year class, and the standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than twice that of the shortest fish. Weights and lengths should be determined by measuring representative specimens before the test or the control fish after the test. Very young fish (not yet actively feeding), spawning fish, and spent fish should not be used.

Daphnia magna used in testing should be in the early instar stages (stages 2-4) of their life cycle. All organisms in a test must be from the same source and as healthy and uniform in size and age as possible.

Culturing, Care, and Handling

Fathead minnows are maintained at 20-22°C in a flow-through system with a turnover of at least two volumes daily, or in a recirculating system in which the water is passed through a carbon filter and an ultraviolet sterilizer (ASTM, 1980).

Daphnia magna are maintained in a static system at 19-22°C. Tanks must be cleaned with a siphon periodically to remove debris, and water should be added as necessary to maintain volume. Cultures must be maintained under optimum conditions at all times to prevent formation of ephippial eggs; daphnids from cultures in which ephippia are being produced must not be used in testing. Generally, periodic subculturing of cultures, elimination of crowding, and adequate food prevent problems in Daphnia cultures.

Both species should be fed at least once a day, at which time careful observations should also be made for mortality and for signs of disease, stress, and injury. Dead and abnormal individuals should be removed as soon as they are observed.

Water quality should be held constant as described above and temperature changes should not exceed 3°C in any 12-hour period. Fish tanks should be scrubbed at least twice a week.

The organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible so that the organisms are not needlessly stressed. Small dip nets are best for handling fish and wide bore pipettes (0.5cm) for Daphnia. Organisms that touch dry surfaces or are dropped or injured during handling should be discarded.

Test organisms should always be shielded from disturbances, and overcrowding should be avoided.

Holding and Acclimation

After collection or transportation, the fish should be held in and acclimated to the dilution water for at least 2 days before beginning a test under the same holding conditions as described in the Care and Handling, section.

A group of animals must not be used for a test if individuals appear to be diseased or otherwise stressed or if more than 5 percent die within 48 hours prior to beginning the test. If a group fails to meet these criteria, they must be discarded or treated and held an additional 4 days.

Fathead minnows should not be fed for 48 hours prior to the beginning of a test. However, the *Daphnia* may be fed up to the beginning of the test.

Test Procedures

Unless the approximate toxicity of the sample is already known, at least six concentrations of test material should be prepared. The highest dose should be at the maximum applicable dose (MAD) for that sample type (see Table A-1) unless physical characteristics of the sample or other previously gathered toxicity data contravenes this.

In fathead minnow tests, at least 20 fish must be exposed to each test concentrations per replicate with two replicates per concentration used in the test. For *Daphnia magna* tests, five organisms per replicate with three replicates per concentration should be used. The use of more organisms and replicate test containers and random assignments of test organisms to containers is desirable.

The fathead minnow tests should be conducted at 22 ±2°C, and those with *Daphnia* at 19 ±2°C. A photo period of 16 hours light and 8 hours dark is used for both tests. Neither type of test animal should be fed during exposure. The test conditions are summarized in Table A-6.

Table A-6

SUMMARY OF TEST CONDITIONS
(from Brusick and Young, 1982)

	Fathead Minnow, *Pimephales promelas*	*Daphnia magna*
Temperature, °C	22 ± 2	19 ± 2
Photoperiod, hours light:dark	16:8	16:8
Water quality, hardness[a] mg/l as $CaCO_3$	100	100
Container size	20 liters	250 ml
Test volume	15 liters	200 ml
Organisms per container	10	5
Replicates	2	3
Feed	No	No
Duration, hours	96	48
Measurements of D.O. and pH, hours	0, 24, 48, 72, 96	0, 48

[a] For dilution water only; the investigators add salts in Table A-5 as appropriate to obtain 100 ug/l as $CaCO_3$.

In the fathead minnow test there should be 15 liters of test solution or control water in each 20-liter jar. If 30 x 30 x 60 centimeter containers are used, the solution should be between 15 and 20 centimeters deep (about 30-35 liters).

In the daphnid test there should be 2 to 3 liters of solution or control water in each 4 liter wide-mouth bottle or 3 to 4-liter battery jar, or 200 milliliters in each 250-milliliter beaker.

Test organisms should be placed in the test and control vessels not more than 30 minutes after the test solutions are prepared. Ten fish in each vessel and five daphnids in each replicate are recommended. Chemical, physical, and biological data are taken and recorded as described below for the duration of the test.

If no toxicity is detected at any concentration and the MAD dose was tested, then no further testing is required. The test material may be reported as having no detectable toxicity. Test materials that kill or immobilize all or nearly all the test organisms at all dilutions should be retested with a lower dose range.

The biological loading in each test and control vessel should not exceed 0.8 g of test organism per liter or be so high as to (1) reduce dissolved oxygen concentration in the control tanks below acceptable levels, (2) raise the concentration of metabolic products above acceptable levels, or (3) stress the organisms by overcrowding, any of which may invalidate the test results.

Results and Data Interpretation

In the fathead minnow test, dissolved oxygen concentration, and pH should be measured for each replicate at the beginning of the test and every 24 hours thereafter in the controls and in the high, medium, and low concentrations. Conductivity and hardness should be measured at the beginning of the test in the control and each test concentration for each replicate. Meters can be used but must be standardized. Temperature of the water bath or controlled-temperature area should be recorded continuously or every 24 hours.

In the _Daphnia_ test, temperature, dissolved oxygen, pH, hardness, and conductivity on the high, medium and low concentrations, should be recorded initially and at 48 hours.

Mortality is the effect most often used to define acute toxicity to aquatic organisms. Criteria for death are usually lack of movement, especially of gill movement in fish, and lack of reaction to gentle prodding.

Because death is not always easily determined with some invertebrates, an EC_{50} may be calculated rather than an LC_{50}. The principal criterion for effect on _Daphnia_ is immobilization, defined as lack of movement except for minor activity of appendages.

Mortality, immobilization, and abnormal behavior should be recorded. Dead or immobilized organisms should be removed as soon as they are observed. For definitions of fish behavior terms, and suggested code for recording and reporting, see Table A-7. If more than 10 percent of test organisms in any control die or are immobilized, the entire test is unacceptable.

The concentration of test material lethal to 50 percent of the population (LC_{50}) and 95 percent confidence limits should be determined at 24-, 48-, 72-, and 96-hour exposures for fish tests, and the EC_{50} and 95 percent confidence limits at 24- and 48-hour exposures for *Daphnia magna* tests. Any of several methods including moving average, Spearman Karber, Litchfield-Wilcoxin, probit, or binomial may be used. For a discussion of the above methods, refer to the review article by Stephan (1977). The results (96 hours for fish and 48 hours for *Daphnia*) are evaluated according to Table A-1 which defines the toxicity categories.

Table A-7

DEFINITION OF FISH BEHAVIOR TERMS
(From Brusick and Young, 1980)

Code	Term	Definition
1.	General Behavior	Observable responses to the test fish, individually or in groups, to the range of factors constituting their environment.
a.	Quiescent:	Marked by a state of inactivity or abnormally low activity; motionless or nearly so.
b.	Hyperexcitable:	Reacting to stimuli with substantially greater intensity than control fish.
c.	Irritated:	Exhibiting more or less continuous hyperactivity.
d.	Surfacing:	Rising and remaining unusually long at the surface.
e.	Sounding:	Diving suddenly straight to the bottom; remaining unusually long at the bottom.
f.	Twitching:	Moving the body or parts of the body with sudden jerky movements.
g.	Tetanous:	In a state of tetany; marked by intermittent tonic spasms of the voluntary muscles.
h.	Flaccid:	Lacking tone, resilience or firmness; weak and enfeebled; flabby.
i.	Normal:	Unaffected by or not exposed to a particular experimental treatment; conforming to the usual behavioral characteristics of the species.
2.	Swimming.	Progressive self-propulsion in water by coordinated movement of tail, body, fins.
a.	Ceased:	Broken off or tapered off to a stop.
b.	Erratic:	Characterized by lack of consistency, regularity, or uniformity; fluctuating, uneven; eccentric.
c.	Gyrating:	Revolving around a central point; moving spirally about an axis.
d.	Skittering:	Skimming hurriedly along the surface with rapid body movements.
e.	Inverted:	Turned upside down, or approximately so.
f.	On side:	Turned 90° laterally, more or less, from the normal body orientation.
3.	Pigmentation.	Color of skin due to deposition or distribution of pigment.
a.	Light discolored:	Color appearance lighter than usual for the species.
b.	Dark discolored:	Color appearance darker than usual for the species.
c.	Varidiscolored:	Color appearance abnormally varied; mottled.
4.	Integument.	The skin.
a.	Mucus shedding:	Observably losing mucous skin coating to an abnormal degree.
b.	Mucus coagulation:	Showing observable clumping or clotting of the mucous skin coating, especially at the gills.
c.	Hemorrhagic:	Visibly bleeding as from gills, eyes, anal opening.
5.	Respiration.	Physical action of pumping water into mouth and out through gills, so as to absorb oxygen.
a.	Rapid:	Observably faster than normal to a significant degree.
b.	Slow:	Observably slower than normal to a significant degree.
c.	Irregular:	Failing to occur at regular or normal intervals.
d.	Ceased:	Broken off or tapered off to a stop.
e.	Gulping air:	Swimming at surface with mouth open and laboriously pumping surface water and air through gills.
f.	Labored:	Performed with apparent abnormally great difficulty and effort.

No Observed Effect Concentration: The highest test concentration in which fish experience no mortality and exhibit no observable behavioral abnormalities at any time during a specified period of exposure to the test material. Ordinarily determined for periods from the start of testing to the end of each successive 24 hours.

MATERIALS AND METHODS FOR BIOLOGICAL TESTS OF SOIL SAMPLES

GENERAL INSTRUCTIONS

Setup and Preparation

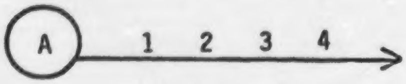
The recommended test organisms in terrestrial tests are seeds from various angiosperms used in the root elongation test (RE test), earthworms *Eisenia foetida*, and soil litter microorganisms. The recommended test period is 115 hours for the RE test, 14 days for the worms, and 14 days for the soil litter test. The principal findings are EC_{50} for the seeds measured by percent germination and root elongation, EC_{50} for the worms, and EC_{50} for the soil litter test. Although inhibition of seed germination and root elongation are observable toxic responses and are reported, root elongation inhibition is the preferred endpoint for the RE test. The concentration which inhibits root elongation by 50 percent of the control (EC_{50}) is estimated and used to rank samples.

Containers, Cleaning, and Preparation

Required containers are discussed under the appropriate test. Cleaning and preparation of test containers should follow procedures described in the GENERAL INSTRUCTIONS for the aquatic tests.

Sampling and Sample Preparation

Samples should be collected randomly at the site boundary or other critical location or along a suspected gradient (identified by any of several methods) such that the most impacted soil can be identified on one end of the gradient and the non-impacted soil on the other end. An example experimental design is shown in the following diagram where A can be a lagoon, an area where leaky drums are stored, or other situation:



A grid at each sampling point is set up, and the surface soil is sampled from a randomly selected quadrant. The points can be uniformly or logarithmically spaced depending on objectives.

Soil samples are returned to the laboratory, and should be analyzed as soon as possible. Storage at 4°C can be used. When tests are to be run, the samples are air dried and ground. If extracts are to be assessed in any of the aquatic or terrestrial tests, they should be prepared all at one time using the procedures in Table A-8. The reason for splitting the extract sample for the various tests is to standardize the procedures so that results may be more comparable among the different tests. The split will then be diluted to yield various concentrations of the extracts for the test. Concentrations should be related to the soil extracted.

Table A-8

METHODS FOR PREPARING SOIL EXTRACT

Steps

1) Weigh an adequate amount of air dried soil sample for all desired tests.

2) Add a weight of distilled water equal to four times the soil weight.

3) Shake for 48 hours (150 rpm) at constant temperature (20 ± 2^0) in the dark.

4) Allow to settle, decant and filter with 0.45 um membrane to obtain the extract. Soil sample extracts with high clay content will have to be centrifuged and decanted prior to filtration.

5) Relate all extracts to the original weight of soil. Measure volume of extract and relate to initial soil weight. For example, if 3100 ml of extract is obtained from a 1000 gram of air dried soil, there are 3.1 ml/gram. Then, if 25 ml of extract are added to 100 gram of soil for a test, this would be equivalent to 8 gram of soil (25/3.1) or a 7.4 percent soil (8/108). This would be the highest concentration and for a geometric series of tests subsequent samples would be decreased by halves. For example, for 7.4, 3.7, 1.85, ... percent, extract plus sample volumes would be: 25 + 0, 12.5 + 12.5, 6.25 + 18.75, ...

6) Do not concentrate extracts; extracts should be prepared within 24 hours of collection. Extracts should be checked for salinity using conductivity.

To perform the soil tests with the earthworms or litter decomposition, the soil samples will be diluted (percent W/W) with artificial soil to produce the desired tests concentrations.

To perform the terrestrial tests, the following soil samples are needed (minimum in parentheses):

	Soil, kg	extract, l
RE Test	2.5 (1.25)	10 (5)
Earthworm Test	4.0 (2.0)	
Soil Respiration Test	2.0 (1.0)	

These samples would provide sufficient material to perform a second test. If range-finding tests or further repeat assays are performed, additional samples should be collected.

ROOT ELONGATION TEST

Introduction and Rationale

The development of a seed into a mature plant is a series of complex processes. To assess toxic effects requires the selection of a stage in plant development that is sensitive to a broad range of toxicants and is important physiologically. Seed germination and root elongation are critical links in plant development beginning with a dormant embryo and during a period of rapid growth when essential plant structures are formed.

Toxic substances that prevent or reduce germination or root elongation will decrease plant populations and can reduce crop yields. In natural systems those species affected are less able to compete with other species and tolerant species may be selected, resulting in changes in species diversity, numbers, and population dynamics.

The inhibition of seed germination and root elongation has been used in determining selective toxicities of herbicides (Horowlitz, 1976; Santelman, 1972)), screening plants for heavy metal (Imai and Siegel, 1973; Walley et al., 1974) and salinity tolerance (Durrant et al., 1974; Neiman and Poulsen, 1971), and evaluating toxic chemicals (Hikino, 1978; Rubinstein, et al, 1975) and allelopathic substances (Asplund, 1969; Muller, 1965). However, many toxicants apparently do not affect plants significantly (Kenaga, 1981), but samples must still be evaluated. The root elongation/seed germination bioassay has several advantages. It is a rapid test germination and root elongation can be observed after 115 hours of incubation. It is a simple test that does not require large expenditures for equipment and facilities or complicated techniques. Personnel required for performing the bioassay do not need to be highly skilled.

The same chemical may cause responses at different doses in different plant species (Rubinstein et al., 1973). To detect an effect from chemicals of unknown toxicity, several plant species should be selected. The species used in this test -- lettuce (butter crunch), Lactuca sativa L.; cucumber (hybrid Spartan valor), Cucumis sativa L.; red clover (Kenland), Trifolium pratense L.; wheat (Stephens), Triticum aestivum L.; and radish (Cherry Belle), Raphanus sativa L. -- are representative of economically important plants and different plant families. Seeds of the selected species should germinate, grow rapidly, contain no natural inhibitors, and require no special pretreatment. All test organisms are grown under identical environmental conditions (constant temperature, 25°C, constant dark, and enclosed to maintain uniformly high relative humidity).

Although inhibition of root elongation and germination are observable toxic responses, root elongation inhibition is the preferred endpoint in this bioassay. Usually, elongation is inhibited at lower concentrations of toxic substances than is seed germination.

Materials and Methods

Facilities

The facilities must include work areas for planting seed and for measurements, preferably isolated from other activities. There should be a fume hood, a distilled water source and refrigeration available at 4°C. The test facility must have a controlled environmental chamber capable of maintaining a uniform temperature at 25°C within ±2.0°C.

Test Containers

One-piece molded-glass tanks (for example, Anchor-Hocking Glass Co., Lancaster, Ohio 43130), with a 6-liter capacity (approximately 24 cm (L) x 16 cm (W) x 18 cm (H)) are used for dosing seeds. Glass plates (13 cm x 15 cm) of single-strength window glass are prepared with polished edges. The glass plates are supported at a 67° angle in the tank with glass pegs. The pegs are 2 to 3 cm long and 5 mm in diameter. Twenty pegs are attached with epoxy to the inside of each glass tank (Figure A-1). An alternative is

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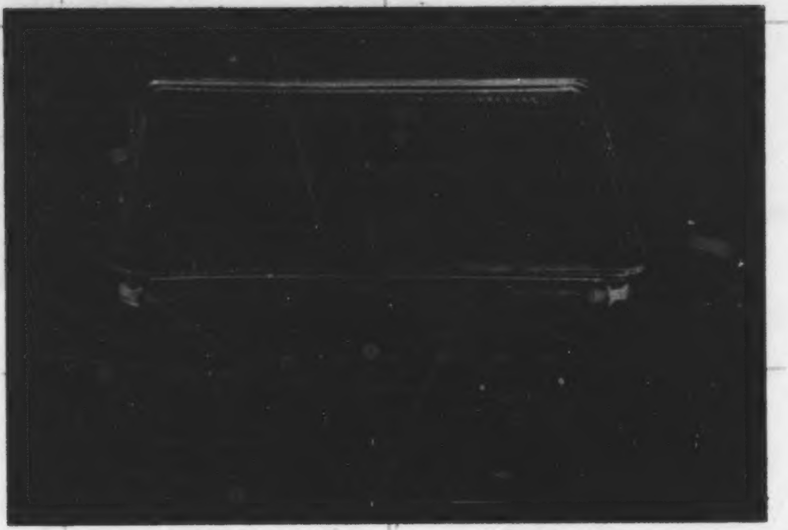
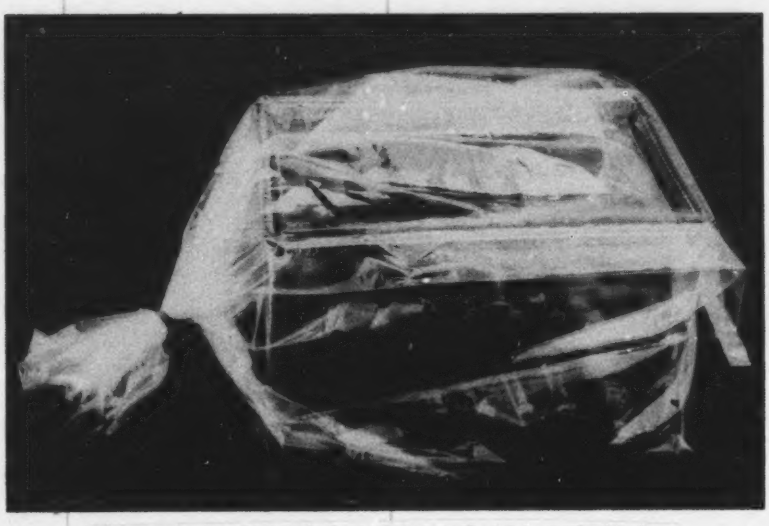


Glass tank with glass plates inserted between rods.



Glass tank with inserted glass plates enclosed in a plastic bag.

Figure A-1. Example Presentation of Setup of Glass Tanks for RE Test.

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a glass rack (for example, Shamrock Scientific Glassware, Little Rock, Arkansas 72205) constructed from two glass rods (approximately 23 cm long) and six half-circles (12 cm O.D) of glass tubing connected to the rods at right angles at 35 mm intervals.

Equipment

Items specifically needed include a spray bottle with a fog or mist nozzle, metric ruler, forceps, Soxhlet extraction apparatus, triple beam balance, pH meter, storage bottles, and plastic bags (minimum of 60 cm x 20 cm x 36 cm). An illuminated magnifier may be helpful for planting, seedling examination, and root measurement.

Test Organisms

The seeds used in the test are available from commercial seed companies, State Agricultural Experiment Stations, and laboratories of the U.S. Department of Agriculture. Seed from one seed lot for each species should be purchased in amounts adequate for 1-year's testing. Information on seed lot, the seed year or growing season collected and germination percentage should be provided by the source of seed. Only untreated (not treated with fungicides, repellants, etc.) seed is acceptable for the bioassessment protocol.

Size Grading of Seed

After purchase, size grading is carried out on the entire seed lot for each kind of seed. Small samples of 100-150 g are sized at a time. The seed lot is inspected and trash, empty hulls, and damaged seed are removed. Depending on species, select a series of four screens to separate sample into size classes (see Table A-9). The four screens are nested with the

Table A-9

HAND SCREENS FOR SIZING SEEDS
(From Brusick and Young, 1982)

	Perforated Metal Sheet		
Species	Round Holes	Oblong Holes	Wire Mesh
Red Clover	1/19, 1/18, 1/17, 1/16 (Fractions of an inch)		
Radish	6-1/2, 7, 7-1/2, 8 (64ths of an inch)		
Wheat	9, 9-1/2, 10, 10-1/2 (64ths of an inch)		
Cucumber		1/13 x 1/2 1/14 x 1/2 1/15 x 1/2 1/16 x 1/2 (fractions of an inch)	
Lettuce			1/6 x 1/28 1/6 x 1/30 1/6 x 1/32 1/6 x 1/34 (fractions of an inch)

[a] Supplied by (for example), A.T. Ferrell and Company, Saginaw, Michigan 48601, or Seedburo Equipment Company, Chicago, Illinois 60607.

screen containing the largest holes on top and screens with successively smaller holes in sequence below. A blank or bottom pan collects the fraction that passes through all screens. Seed is poured onto the top screen and the whole set of nested screens are shaken (by hand or with a vibrator) until all the seed remains on one screen or reaches the bottom pan. The separated fractions are collected and saved. The procedure is repeated until all the seed in the lot is sized. That size class which contains the most seed is selected and used exclusively for the duration of the tests. The seeds in the size class are divided into small lots, placed in separate envelopes or sacks, and stored in moisture-proof sealed containers in a refrigerator at $5^{o}C$.

Preparation of Glassware

The glass tanks (fitted with glass pegs or tanks with glass racks) and glass plates are thoroughly washed as described under Cleaning and Preparation of Glassware in the aquatic section.

Tissue Paper Precleaning

Eight to 10 sheets of single-ply cellulose tissue (for example, Kimwipes) are placed in a Soxhlet Extractor and extracted using standard chemical procedures with distilled water for a minimum of 24 hours (4 cycles/hour). After extraction, the tissues are removed, air dried, and stored in a dry glass container.

Test Procedures

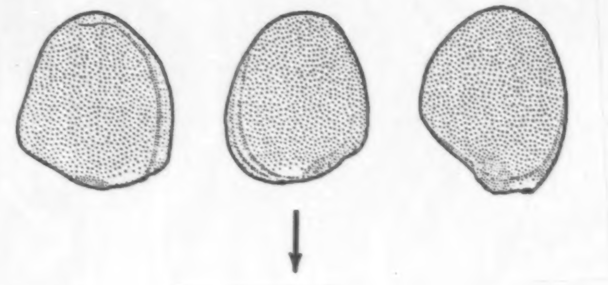
Test Medium

The test medium is an aqueous extract of a particulate or solid sample. Aqueous extracts of solids are prepared using the procedure outlined in Table A-8. Aqueous extractions of solid samples should be tested as soon as possible or the solid sample must be stored in closed polyethylene containers until extraction can be made. Dilutions of the extract or aqueous extractions should be made without use of solvents or additives except for distilled water, which is used as a negative control. Acids and salinity will cause toxicity in some cases. Generally, extracts with pH greater than 6.5 and salinity lower than 0.01 N salt will not be toxic.

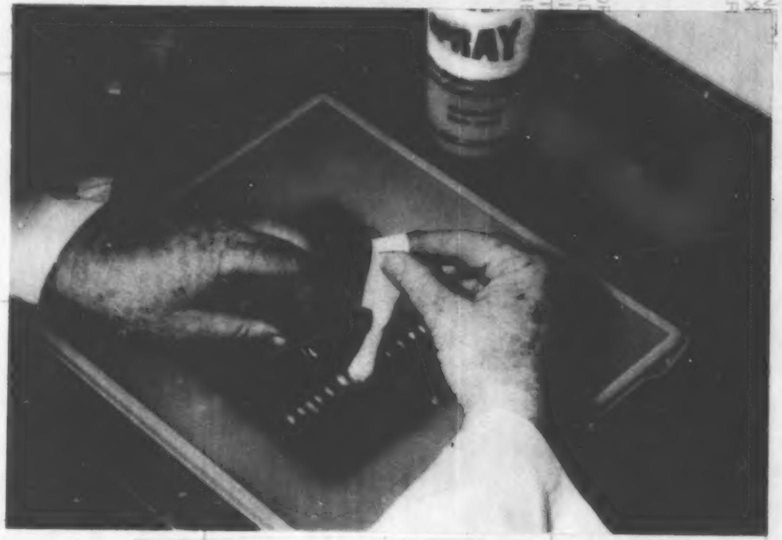
Acid and saline soils may exert seed elongation toxicity even without toxicants and it may be necessary to compare results to a artificial control.

Procedure for Planting Seed

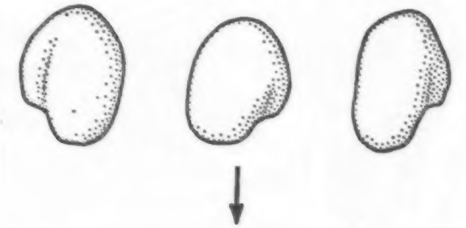
Whatman No. 3MM chromatography filter paper rectangles (13 x 15 cm) are soaked in the test solution in a shallow tray for a minimum of 5 minutes to saturate. One sheet of filter paper is removed from the test solution, allowed to drain, and placed on a glass plate to which the paper adheres. Trapping air bubbles between the filter paper and the glass plate should be avoided. Using forceps, 15 seeds from one species are placed on the filter paper substrate in a row, equally spaced, across the top of the plate 2.5 cm down from the top edge. Seeds are placed with the radicle end (embryo or germ) toward the bottom of the plate (Figure A-2) and, in the case of wheat, with embryo side of the seed up. A narrow strip, (1/2 cm wide) of previously cleaned (Soxhlet Extraction) single-ply tissue is placed over the row of seeds to hold them in place and, if necessary, sprayed with just enough fine distilled water mist to cause the tissue to cling to the seeds and filter paper. Test solution, usually 500 ml, is poured into the rectangular glass tank fitted with glass peg guides (empty tank if glass rack is used). The glass plate holding seed and substrate is inserted in the glass tank between the glass peg guides or in the glass rack to support the plates at a 67° angle with the horizontal (Figure A-1). The lower end of the plate opposite the seeds should be immersed in the test solution with a minimum of 2 cm, but not more than 3 cm, of the plate and filter paper in the solution. Solution volumes smaller than 500 ml can be used if clean inert glass beads are added to the solution to displace and raise the liquid level. This procedure is repeated for each seed type (lettuce, radish,



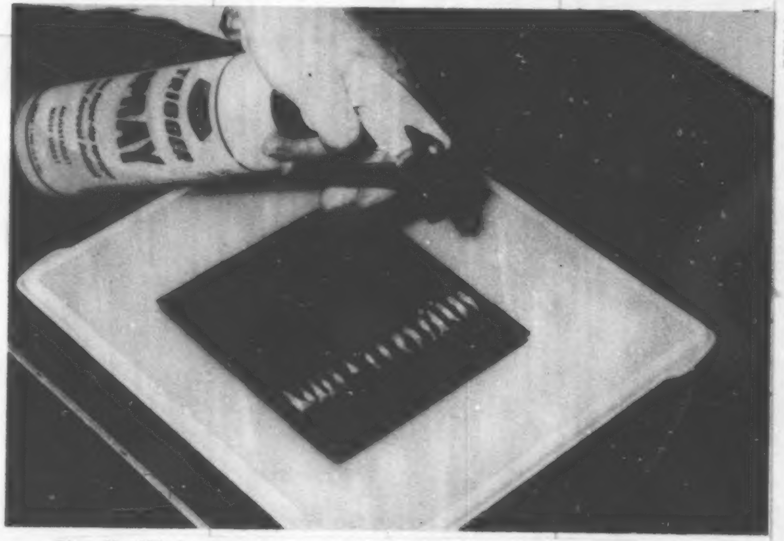
Radish seed (enlarged) with radicle end toward the bottom of plate.



Seed being covered with narrow strips of tissue paper.



Red clover seed (enlarged) with radicle end toward bottom of plate.



Fine distilled water mist causes tissue to cling to seed and filter paper.

Figure A-2. Examples of Preparing and Orienting the Seeds for the RE Test.

wheat, cucumber, red clover).

Incubation

The glass tank containing 5 plates with 15 seeds each and the test solution is enclosed in a heavy plastic bag and tied shut (Figure A-1). The enclosed tank is placed in the dark, $25\pm2^{\circ}C$ controlled chamber. A tank is prepared for each test solution of sample solution, the positive controls, and the negative (distilled water) controls. The concentration range of NaF for the positive controls which causes an EC_{50} for each seed species is: radish 400-500 mg NaF/l; wheat 300-400 mg NaF/l; lettuce 100-200 mg NaF/l; cucumber 150-200 mg NaF/l; and red clover 80-100 mg NaF/l.

Measurement of Root Length

Measurement of root length is made at 115 hours from the start of dark incubation. It is important to measure each plate as nearly as possible to 115 hours (not to exceed ±30 minutes). To measure root length, remove a plate from the tank and place it on a flat surface. The lengths of all roots are measured to the nearest millimeter and entered on the data sheet. Measure from the transition point between hypocotyl and root to the tip of the root (Figure A-3). At the transition between the hypocotyl and the primary root the axis may be slightly swollen, contain a slight crook, or change noticeably in size (radish, lettuce, cucumber, red clover). In wheat, the single longest primary or seminal root is measured from the point of attachment to the root tip. For additional descriptions and photographs helpful in making root measurements, see USDA (1952) and Wellington (1961).

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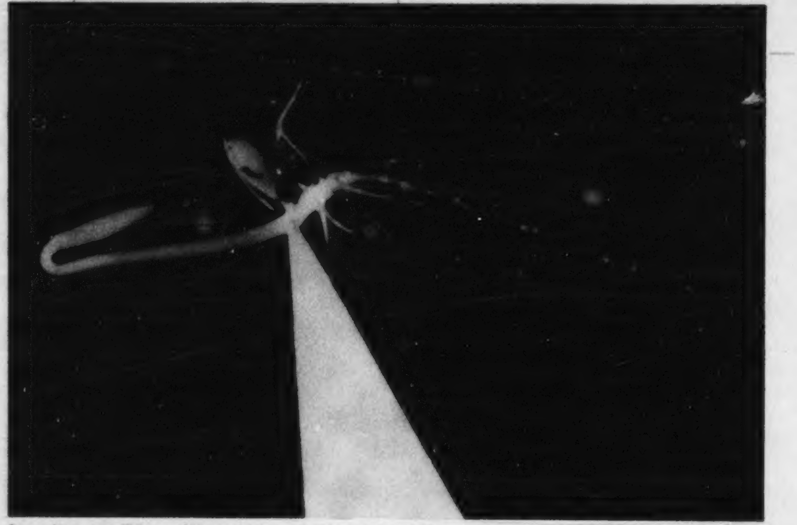
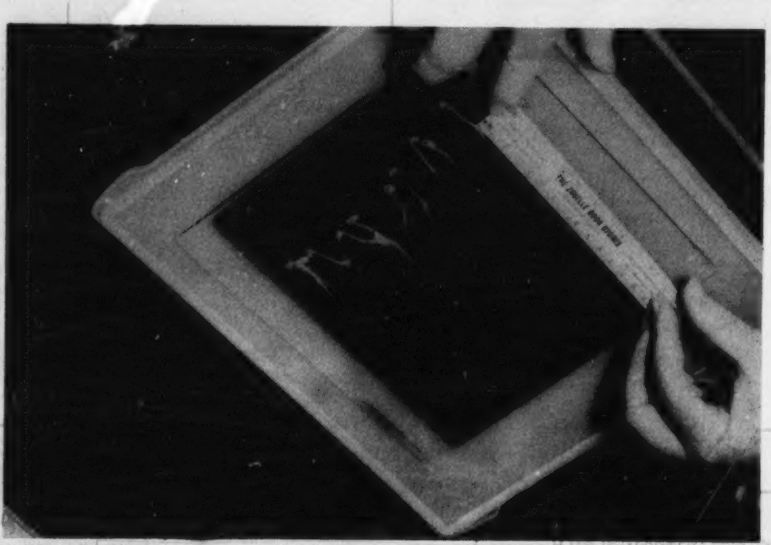
Radish seedling with pointer showing transition point between root and hypocotyl.



Cucumber seedling with pointer showing transition point between root and hypocotyl.



Seedling showing method of root measurement using metric ruler.

Figure A-3. Examples of Measuring Root Elongations in the RE Test (Brusick *et al.*, 1980).

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Range-finding Test

The purposes of the range-finding test are to determine if definitive testing is necessary, and to aid in the selection of concentrations to be used in the definitive test when needed. The range-finding test consists of one control tank, and one tank each of 100, 10, 1, 0.1, and 0.01 percent extract.

A species need not be included in the definitive test if the tank containing 100 percent extract had mean root lengths of at least 65 percent of control and at least 10 of 15 seeds germinated. Also, in this situation it is not necessary to examine the plates containing this species in the 10 to 0.01 percent tanks. If one or more of the species show mean root lengths less than 50 percent of the control at even the most dilute concentrations, it is advisable to extend the range and repeat the range-finding test before proceeding to the definitive test.

Definitive Test

Estimation of an EC_{50} in this test will require a control and at least six extract concentrations chosen in a geometric series. The highest concentration in the definitive test should be the next concentration greater than the range-finding concentration which reduces mean root length to less than 50 percent of the control. For example, if the range-finding test shows that 1 percent extract causes mean root lengths less than 50 percent of the control, then the definitive test would begin at 10 percent extract. In a geometric series, the ratio of one concentration to the next is the same: for the above example, 10, 5, 2.5, 1.25, 0.615, and 0.312 percent. If more than six concentrations are used, not all species must be tested at all concentrations. However, each species must be tested with at least six concentrations and those concentrations must be in a geometric series.

Results and Data Interpretation

Assay Acceptance Criteria

To accurately estimate the EC_{50}, specific criteria must be met for each of the test species. For the definitive test, criteria 1, 2, and 3 must be met:

1. At least 10 of 15 seeds on the control plate must germinate.

2. Each test concentration in a series must be at least 50 percent as strong as the next concentration, except for the control.

3. Four toxic concentrations must be defined in the definitive test for which mean root length is inhibited in the following ranges: less than or equal to 25 percent, less than or equal to 50 but more than 25 percent, more than 50 but less than or equal to 75 percent, and the lowest concentration which is more than 75 but less than or equal to 90 percent.

Since most toxicants affect root elongation at lower concentrations than germination, criterion 3 must be met to satisfy the requirements of the definitive test in addition to criteria 1 and 2. If a species fails to satisfy criteria 1, 2, and 3, the definitive test must be repeated for that species.

Calculations and Reporting

Provided criteria 1, 2, and 3 are met in the definitive test, the EC_{50} is estimated in the following manner. For each species which satisfied these three criteria, plot on semi-log paper sample concentration on the logarithmic axis and percent control mean root length on the arithmetic axis. Draw a straight line between the four test sample concentrations used to satisfy criterion 3. The concentration at which this line crosses the 50 percent point for the control root length is the EC_{50} for root elongation. If no effects were seen with 100 percent sample, or if criterion 3 could not be met due to germination inhibition, it is not possible to estimate an EC_{50} for root elongation.

For each of the species tested either the concentration in (a) or (b) must be calculated and reported.

(a) If the species satisfied criteria 1, 2, and 3, report an estimated EC_{50} for root elongation. Use graphical interpolation to estimate the EC_{50} and rank the test sample using the evaluation criteria in Table A-1.

(b) If the species satisfied criteria 1 and 2 but not criterion 3 (criterion 4 or 5 used instead), report the lowest concentration for which fewer than 10 of 15 seeds germinated. The EC_{50} cannot be estimated for root elongation or inhibition of seed germination from data in this category. Currently, test samples are not ranked from data of this type.

EARTHWORM TEST

Introduction and Rationale

Earthworms have been selected as an indicator species because they are representative of the terrestrial environment and are of considerable importance in improving soil aeration, drainage and fertility (Edwards and Lofty, 1972). The tests developed in this protocol were taken from the European Economic Council Guideline for Testing of Chemical Toxicity to Earthworms (OECD, 1981).

Earthworms differ from aquatic organisms in that they may be exposed to toxic chemicals in the aqueous phase via soil moisture, in the vapor phase, or by coming into contact with particulate matter on the surface of soil constituents. Moreover, they may be protected in soil because many chemicals become tightly adsorbed onto soil fractions, particularly organic matter, and the soil colloids making up the clay fraction.

Hence, a simple immersion test, which yielded consistent and reproducible results for relatively soluble chemicals or formulated pesticides, was rejected because it would not provide information on comparatively insoluble compounds which affect the worms only when they are in direct contact, or on compounds which affect the worms only as a vapor.

There are tests which involve the injection of test chemicals either into the pharynx or body of the worms and although these give reproducible results, they require considerable expertise and have the drawback that it is difficult to relate the results of such tests to field conditions.

The test method is proposed as a two-stage test. The first stage would be a relatively simple contact toxicity test involving exposure of the worms to extracts on filter paper to examine potential toxicity. Toxic samples would then be tested further using soils or applications of extracts in a defined soil medium. The contact test was chosen because the exposure of the worms in such a test more closely resembles the natural situation.

To provide a routine test for the protocol, a commonly used test species was selected. Eisenia foetida is not a common species in soil although it does occur in soils with considerable organic matter. It is common in sewage beds, particularly in trickling filters, where it is exposed to industrial chemicals. It is a species with a short life cycle, reaching maturity in seven to eight weeks at 15-20°C. It is prolific; a single worm produces 2-5 cocoons per week each of which will give several worms. It can be bred readily in a wide range of organic wastes. This means that laboratories could easily breed their own stock if supplied with cocoons from a central source, and a standard strain could be used.

Materials and Methods

Test Organisms

Test organisms should be adult Eisenia foetida (at least 2 months old with a clitellum) of weight 400 - 800 mg. All worms for a specific test should be from the same breeding box. Individual worms are used (1/vial) in the range-finding test; ten individual worms (about 4 to 8 g) should be added to each test container for the definitive test.

Breeding of Test Organisms

Eisenia foetida can be bred in a wide range of animal wastes. The recommended breeding medium is a 50:50 mixture of horse manure and peat, but other animal wastes are also suitable. The medium should be of pH about 7.0, have low conductivity (less than 6.0 umho/cm) and not be contaminated excessively with ammonia or animal urine. Wooden boxes 500 x 500 x 15 cm with tightly fitting lids are ideal for large-scale breeding and should produce more than 1000 worms in six weeks. To produce sufficient worms, such a medium will support 1 kg worms in 20 kg waste and each worm will weigh up to 1 g. To obtain worms of standard age and weight it is best to start the culture with cocoons which take three weeks to hatch and seven weeks to become mature worms at 20°C.

Test Procedures

Range-Finding: Contact Test

Glass vials, 8 cm long x 3 cm diameter are recommended. The sides of these are lined with a strip of filter paper 9.5 x 6.7 cm (Whatman Grade 1).

The extract is applied in water as appropriate, to give a range of known concentrations. The control should be treated with distilled water only.

It is recommended that the toxic dose range be established in a preliminary test after which a more precise test may be made with a restricted dose range. The doses are calculated in terms of volume of extract diluted with distilled water to give the following concentrations: 100 percent, 10 percent, 1 percent extract.

For a more precise contact test, five doses in a geometric series (e.g. in the ratio 100, 50, 25, 12.5, 6.25) should be used. For each test, ten replicates per dose, of one worm per vial, would be the minimum requirement. Do not use more than one worm per vial.

In each test, a range of doses of extract plus a positive control using 0.354 mg Cu/l copper sulfate. This concentration will provide a response range of 0.9 to 1.1 of the LC_{50}. A negative control should be used.

Vials should be laid on their sides for the duration of the test.

Test temperature = 20 $\pm 2^{\circ}$C.

Test in continuous dark.

Test duration = 48 hours.

Worms should be classed as dead when they do not respond to a gentle mechanical stimulus to the anterior end.

Discard vials after the test.

Definitive Test: Artificial Soil Test

In this test, worm survival is evaluated after 14 days in a mixture of an artificial soil (Table A-10) and soil samples or extracts of soils from the site. Extracts can be added to the artificial soil to aid in defining concentrations to test using artificial soil plus soil samples. Otherwise the definitive test can directly be applied using mixtures of site soil and artificial soil. Soil is a variable medium so for this test a carefully defined artificial loam soil is used. It was developed specifically as a growth medium for the earthworm test. The medium for the definitive test should be based upon the three general constituents listed in Table A-10; sieve and chemical analyses are not required. This artificial soil mixes well and *Eisenia foetida* will survive in it for long periods. Its absorptive capacity is similar to that of a typical arable soil.

The dry constituents are blended in the correct proportions and thoroughly mixed mechanically in either a large-scale laboratory mixer or small electric cement mixer. The peat is finely ground in a laboratory mill, and the pH is adjusted to 7.0 by addition of appropriate amounts of calcium carbonate. Moisture content is then determined by drying a small sample at 80°C and reweighing. From these data, the amount of deionized water required to achieve a moisture content of 20 percent of dry weight is calculated (25 g water per 100 g of dry soil). This is added and the medium remixed before use.

Table A-10

COMPONENTS OF ARTIFICIAL SOIL

General Composition by Weight

1. 70% Industrial Sand
2. 20% Kaolinite Clay
3. 10% Sphagnum Peat

Specific Composition

1. Industrial Sand

Diameter in Microns	Percent
45	1.7
45	9.3
63	29.0
90	34.3
125	20.8
180	4.0
250 & greater	0.8

2. Kaolinite Clay

Composition	Percent
SiO_2	58.5
TiO_2	1.3
Al_2O_3	28.0
Fe_2O_3	1.0
MgO	0.3
CaO	0.2
K_2O	2.0
Na_2O	0.3
loss on ignition	8.4

Test Conditions for Artificial Soil and Soil Sample Extracts

A range of dosage levels identified from the contact test results will be used in these tests. As far as possible, the range of doses selected should be chosen so that some are on either side of the estimated LC_{50}. This is expected to bracket the middle concentration (i.e. LC_{50}) of the definitive test and other concentrations in the series would be selected accordingly. For example, if the contact test indicated that the median lethal concentration was in the area of 40 percent of the extract (4 parts extract to 6 parts deionized water), the range used would be 0 (negative control), 20 percent, 40 percent, 60 percent and 80 percent. For a total of 100 parts, the sum of extract and deionized water (V/V) would be 0 + 100; 20 + 80, 40 + 60, 60 + 40, and 80 + 20, respectively. The test containers are 500 ml crystallizing dishes, containing 400 g of the artificial soil, covered with plastic lids, petri dishes, or plastic film.

In each test, one positive control with copper sulphate at a concentration of 600 mg Cu/kg of prepared soil and four control containers treated with solvent blank (distilled water) should be used. Mortality should be assessed by emptying the soil into a tray, sorting out the worms and testing their reaction to a mechanical stimulus to the anterior end. For each test, four replicates per dose with ten test worms should be used. The average weight of test and control worms should be calculated at the beginning and end of the test.

Test Conditions With Artificial and Sample Soils

Soil samples collected from the site must be prepared for mixing and "dilution" with the artificial soil. Soil is prepared by the procedures outlined in steps in Table A-11. Then, using a top loading balance, appropriate amounts of site soil and artificial soil are weighed to prepare the amount of soil needed for the appropriate tests.

Test containers are 500 ml crystallizing dishes covered with plastic lids, petri dishes, or plastic film. In each dish 400 g of the moist test medium is used. For each test dose, a 1600 g mixture of the moist, prepared, artificial soil and freshly sampled soil is prepared. For example, the test concentration desired is 75 percent. Therefore 400 g of moist artificial soil is added to 1200 g of sample soil and thoroughly mixed. Then, four 400 g aliquots are weighed out and placed in each 500 ml crystallizing dish test container. For practical reasons, the sample soil concentration should never exceed 75 percent. Range finding test concentrations could be 0.75, 0.15, 0.075. Definitive test concentrations could be 0.75 0.5, 0.25, 0.125, 0.0625, 0.03125.

Copper sulfate is the positive control and should be included in the soil assay by adding a total of 0.96 g Cu with the deionized water to achieve 600 mg Cu/kg of moist soil. The main purpose of the positive control is to account for variability in the test organisms.

For each test, four replicates of 400 g, each containing ten test worms should be used. A positive and negative control (100 percent artificial soil), each with four containers, should be used. Mortality should be

Table A-11

PROCEDURE FOR HOMOGENIZING SOIL SAMPLES

(Lighthart, 1980; Unpublished Procedure)

1. Air dry soil to be tested. (Air drying is considered completed when an aliquot of soil has no more weight loss.)
2. Add 25 burundum cylinders and about 2 liters of air dried soil to a ball mill.
3. Mill to coffee ground size (ca. 5 minutes) then sieve through a 2mm mesh sieve.
4. Return larger particles back to the ball mill and repeat steps 2 through 4 until the sample is completely ground with the exception of rocks. Discard rocks.
5. Homogenize soil using a laboratory or small cement mixer thoroughly before use.
6. Clean the ball mill by adding 1 quart of silica sand and 10 burundum cylinders. Mill for 15 minutes, discard, and then brush out mill.

assessed by emptying the soil into a tray, sorting out the worms and testing their reaction to a mechanical anterior stimulus.

The average weight of the test and control worms should be determined at the beginning and end of the test. Environmental conditions should be:

The test temperature = 20 ±1°C.

The soil moisture should be 20 ±5 percent

(add 25 ml deionized water/100 g of air-dry soil).

Test in continuous light.

Test duration = 14 days.

An assessment of mortality at 7 days and continuation of test to 28 days is optional. If more than one mortality assessment is made it may be necessary to adjust the moisture content of the soil due to losses during sorting.

Results and Data Interpretation

The mortality/dose data should be plotted on log probit graph paper and the median lethal concentration (LC_{50}) and its confidence limits estimated. If the LC_{50} cannot be established the LC_0 and LC_{100} values should be given.

Mortality in negative controls should not exceed 10 percent. If there is some mortality a correction based on Abbott's formula can be made:

$$\text{Corrected mortality \%} = \frac{\text{Observed mortality \% - control mortality \%}}{\text{100 - control mortality \%}}$$

The LC_{50} values should be given as percent of sample soil (W/W).

SOIL RESPIRATION

Introduction and Rationale

Soil/litter microcosms can be used to define the impact of pollutants upon primary productivity in terrestrial ecosystems (Lighthart, 1980). The measurement of evolved CO_2 from microbial respiration in these microcosms can indicate the degree of pollutant stress within the system. Low CO_2 levels indicate high stress whereas no significant change or an increase in CO_2 can identify low pollutant stress or even stimulation of microbial activity.

A simple soil/litter microcosm toxicity test can be conducted within 14 days. Soil micro-organisms are exposed to various concentrations of the test materials (soil and/or soil extracts) at standard moisture and temperature conditions. Evolution of CO_2 is measured at predetermined time intervals throughout the test. Results are expressed as percent inhibition (EC_{50}) or stimulation (SC_{20}) between CO_2 evolved in control and amended microcosms at specified time intervals.

Materials and Methods

Glassware includes one quart regular Mason (one liter) jars with air tight lids and one ounce (30 ml) glass bottles with air tight lids. These should be washed according to GENERAL INSTRUCTIONS for the aquatic tests.

Triplicate microcosms for each treatment are prepared by placing 100 g of air-dry artificial soil, or combinations with test soil (Table A-11) sieved to pass a 2mm screen into each of three Mason jars. Then, 20 ml of deionized water is added for appropriate moisture conditions in the microcosm. Finally, a one ounce glass bottle with CO_2 trapping solution is added and the air-tight Mason jar lid is sealed securely. A special blank must be used to correct for atmospheric CO_2 during titration. This consists of 3 clean Mason jars without soil and CO_2 trap but which are run concurrently with controls and test jars.

Equipment should include a constant-temperature room or incubator capable of providing temperature control of 20 $\pm 2^{\circ}C$. A ten liter capacity ball mill with 25 3.2 x 3.2cm (1-1/4") burundum cylinders and a 2mm mesh sieve. Standard laboratory equipment such as balances, pH meters, pipettors, magnetic mixers and bars, drying ovens, and appropriate glassware necessary to prepare reagents and perform the titration of CO_2 are also needed.

Test Procedure

The test material is introduced into the microcosm either as a soil or soil extract. The soil sample may be added directly (100g) or by weight percentages as in the earthworm test and added to the artificial soil. The aqueous test extracts are introduced into the artificial microcosms on a percent basis (V/W), i.e. 100, 50, 25, 12.5, 6.25, 3.125, where 100 percent represents 25 ml of soil extract and further dilutions of extract are made with deionized water (Table A-8). The extracts and acid traps are added after two days of incubation at 20°C in the dark if extract is to be studied.

Respiratory carbon dioxide is measured in the alkali traps twice weekly for the duration of the test which is typically 2 weeks.

Three replicate microcosms are required for each control and test (soil and/or soil extract) concentration. Each microcosm is incubated at 20 ±2°C in the dark for 14 days. The evolution of CO_2 is measured twice weekly by titration. Reagents and titration procedures are outlined in Table A-12.

Results and Data Interpretation

The total CO_2 produced during the 14 day test is obtained by summing individual CO_2 measurements for each interval. Percent inhibition (I), or stimulation (S), is calculated after 14 days for each test concentration according to the following formulas:

$$\%I = \frac{C - T}{C} \times 100$$

$$\%S = \frac{T - C}{C} \times 100$$

where C is the mean CO_2 evolution in the control and T is the mean growth in the treated microcosm. Three endpoints are calculated from the percent response vs. concentration data. For samples which are inhibitory, an EC_{50} (defined as the lowest test concentration causing growth inhibition of 50

Table A-12

PROCEDURES OF TITRATING CO_2 IN TRAPS AND METHODS FOR PREPARING REAGENTS

Taken from Lighthart, 1980; Unpublished Procedure)

A. CO_2 Titration Procedure

a. Replace the CO_2 traps at the designated intervals by opening the microcosm and removing the exposed CO_2 trap and replacing it with an unexposed one. (At the same time this step is being performed, insert an open vacuum line to aid in properly replenishing the air in the microcosm. Remove at least 3 times the volume of the air space.)

b. As quickly as practical, place an air tight cap on the exposed CO_2 trap; return the microcosms to the 20°C dark incubator.

c. Add five ml of 1.3N of $BaCl_2$ and a stir bar to each exposed CO_2 trap immediately prior to titration.

d. Titrate excess 0.6N NaOH remaining in the trap to pH 9.0 with a buret and pH meter (or autotitrator) using Trizma standardized 0.6N HCl to measure milligrams of CO_2 produced.

Formula for the Calculation of CO_2 Production:

mg of CO_2 = (Blank ml - Sample ml) x 22 mg of CO_2/ml/N x Normality of Acid

e.g., mg of CO_2 = (10.40 ml - 6.93 ml) x 22 mg of CO_2/ml/n x 0.6013 N

= 45.90 mg of CO_2 produced

B. Preparation of Reagents

1. 0.6 NaOH

a. Rinse 20 liter glass carboy with distilled H_2O.

b. Place on a large magnetic stir plate; add degassed distilled H_2O to the 18.9 liter mark.

c. Add 454 grams (1 lb) of NaOH pellets.

d. Stopper and stir overnight before use. (Maintain the NaOH stock solution in a CO_2 free atmosphere by using ascarite traps.

2. 0.6N HCl

a. Rinse 20 liter glass carboy with distilled H_2O.

b. Add 1.0 liter of concentrated HCl.

c. Add distilled H_2O until the 20 liter mark.

d. Stopper and stir overnight.

e. Titrate 5 "Tris" samples (0.5 to 0.9 grams of "tris" in 10.0 ml of distilled H_2O and 5 ml of 1.2N $BaCl_2$) to pH 5.0 with ca. 0.7N HCl; calculate mean and standard deviation ("s"). (If "s" is larger than 0.0015, do 5 more samples and combine results.)

$$\text{Normality of HCl} = \frac{(0.1211\text{ g/meq})\ (\text{ml of HCl used})}{(\text{Weight of Tris in grams})}$$

$$\text{e.g., Normality of HCl} = \frac{(0.1211\text{ g/meq})\ (9.69\text{ ml})}{0.7089\text{ grams}} = 0.6041\text{N}$$

Table A-12 (Continued)

3. 1.3N $BaCl_2$
   a. Weigh 317.56 grams $BaCl_2:2H_2O$.
   b. Dissolved in degassed distilled H_2O in a 1 liter volumetric flask.

4. Tris
   Aminomethane(hydroxymethyl)tris--Trizma Base (Sigma Chemical Company, St. Louis, Missouri).

percent relative to control) is calculated. For samples which are stimulatory, and SC_{20} (defined as the lowest concentration causing growth stimulation of 20 percent relative to control) is calculated. Also, the measurements of CO_2 made a other times can be used to evaluate anomalous results and to observe time trends of CO_2 production.

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APPENDIX B

GUIDANCE ON SAFETY PROCEDURES FOR WORKING WITH SAMPLES FROM HAZARDOUS WASTE SITES USING THE BIOASSESSMENT PROTOCOL

GUIDELINES AND CONCEPTS OF SAFE PROCEDURES

The objective of these guidelines is to protect workers, the public, and the environment, and to insure that contamination does not occur and interfere with valid laboratory results. The major factor in providing this protection is the common sense of the staff performing the bioassessments. The guidance presented herein is designed to complement this common sense. For example, it is extremely important that all staff follow good housekeeping procedures and maintain personal grooming and cleanliness within the confines of the laboratory area. There are safety courses that are available through OSHA and these should be taken wherever possible. Also there is access to experienced personnel within states, regions, or local communities. These should be drawn on prior to starting and whenever any possible hazards might occur that were not considered.

Whichever safety procedures are utilized, they must be commensurate with the hazard, and hazard depends on the concentration and types of materials that will cause exposure. Hopefully, explosive, ignitable, corrosive, or otherwise highly reactive samples will not be evaluated in the bioassessment protocol. The intent of the protocol is to assess the acute toxicity of soil and water samples. Sample size should be adequate for needs and not in great excess as disposal then becomes more of a problem.

A key factor is the safety committee to oversee procedures written in advance of the setup of the laboratory facilities. This safety committee should include at least a chemist and a biologist and, whenever possible, these persons should have experience in hazardous wastes. In the absence of such experience, a state or other local expert should be utilized.

The facilities are a key part in maintaining the integrity of the protective plans. Wherever possible, separate self-contained facilities should be utilized. For example, field equipment should be labeled for hazardous waste site use only. Disposable sample containers and protective gear and equipment should be used where possible. Solid and liquid waste handling procedures should be specified and materials should be placed in unbreakable containers that are easily transported. A separate storage area a separate preparation room, and a separate experimental area should be required. All of these facilities should be lockable and maintained under safe and secure conditions.

Air should be supplied using forced air fans and complete exchange of the air supply in the preparation area should occur on an average of once every five minutes at the minimum. Air supply in general should be commensurate with potential hazard and the cost of providing air exchange. Chemical fume hoods are an excellent means of providing this kind of safety for sample preparation.

Personnel are protected with respirators, gloves, and laboratory clothing which are disposable or, in the absence of severe exposures, washable. Laboratory services should be isolated with appropriate check valves and/or supply services. This can include air supply, water supply and gas supplies. Vacuum services should be entirely separate; small vacuum pumps can provide adequately for most cases.

Finally, personnel involved should be clearly identified and their utilization of the laboratory facilities involved with hazardous waste materials should be controlled and recorded. Medical surveillance should be implemented where needed. In any case, a complete physical examination with chemical measurements of blood and urine samples should be implemented prior to any work in the laboratory area with known hazardous materials. One should avoid the perception as well as the reality of risk. Specific procedures are identified in the following paragraphs.

1. Work schedule and procedures. All work to be performed should be detailed in advance and written out for all personnel involved and for review by the safety committee. A responsible person should be designated the hazardous waste material disposal officer. Detailed procedures on handling of soil and water samples, dilution procedures in water and synthetic soils, disposable and unbreakable containers, storage access, analytical measurements, and pertinent information should be written out in advance of any experiment. Standard procedures should be followed to minimize the paper work involved. However, all personnel involved must sign a form stating that they have read and understood the instructions. A simple test designed to determine their understanding of the procedures can be maintained in the personnel file.

2. Sampling, handling and storage. Sampling should be done using careful procedures since actual sites will have more hazardous exposure than will the laboratory facilities. In some cases, it will be wise to subcontract to a firm specializing in hazardous waste handling in order to collect samples. Designated, separate field sampling equipment should be

utilized for collecting water and soil samples.

Samples should be stored in disposable, non-breakable sample containers. Metals should be placed in polyethylene containers. Organics should be collected and stored in disposable glass bottles and bottles packed in absorbent material that can account for the entire liquid in the sample. Labels should be affixed to all samples.

3. Personnel. Personnel who are allowed to have access to the sampling and laboratory facilities should be identified clearly. Personnel testing or monitoring should be performed and recorded. For each type of waste, the need for medical surveillance should be evaluated. Personnel should be medically tested whenever especially hazardous conditions occur.

5. Chemical information form. All available information on the chemicals that are potentially present at a site should be accumulated. This information will be invaluable in terms of analyzing potential environmental hazards that exist at a site for the protocol as well as protecting the personnel.

6. Identification of the potentially most hazardous operations. It is important that all operations be written down as in item 1 above. The most potentially hazardous operations should be identified in this section and described in detail. After identifying these operations it is important to explain what

procedures should be followed during potential accidents and/or routine safety procedures. Each operation that may be included in this section should be carefully identified and discussed with appropriate clean-up and disposal procedures.

7. Accidental exposure and emergency treatment requirements and procedures. Based on the chemical list and the potentially most hazardous operations, appropriate procedures should be spelled out. Monitoring for potential health problems that might occur should be detailed in this section.

8. Accidental release information. Accidental releases from bioassessment procedures are probably not a critical factor. However, a chain of custody form (see Section 16) must be used for all sample handling so that storage, utilization, dilution, and ultimate disposal by the laboratory disposal officer will be recorded for future use.

9. Waste disposal procedures. Al toxic materials, original samples and high dilutions of samples, must be packaged in unbreakable containers and deliverable to the disposal officer. For safety and public relations reasons it is important to dispose of all contaminated materials in a safe manner to a hazardous waste site. Segregation of protective clothing and samples that are in the low response levels into one category and intermediate to high response dilutions and actual samples into a high level category will aid disposal operations. It is the responsibility of the disposal officer

to dispose of these using approved state procedures or other applicable regulations.

10. Personnel protection. Protective clothing including laboratory coats, and shoes, covers, eye protection, gloves, and face protection should be specified where necessary. In addition, respirators and dust filters generally should be utilized in the preparation room and also where hazards suggest that it is necessary in the experimental area. Each individual should carefully wash in a secure area that is outside of both the preparation room and the laboratory experimental area. Soap and water are usually adequate for washing. However, it is important that the protective clothing be utilized in the preparation room and discarded at the door in safe containers before exiting to the wash area.

11. Signs. The laboratory facility should be isolated from general public contact. Authorized personnel should be the only persons allowed in the experimental area. Warning signs should be posted and controlled access should be maintained at all times.

12. Work area identification and access control. Although signs are necessary for information reasons, it is important that all areas be locked and public access kept under surveillance and minimized. The storage area in particular should have double locking procedures with a signature form and chain of custody form for samples.

13. Work surface protection. Preparation rooms and laboratory facility areas should be covered with disposable plastic backed absorbent paper.

14. Contaminant devices. All samples should be stored in sealed containers in the locked storage area. Subsamples can be prepared in the preparation room and samples returned to the storage area. After an assay, all samples should be delivered in disposable containers to the disposal officer.

15. Storage. The storage of samples should be minimized where possible. Only enough sample to meet the needs of the bioassessment should be collected plus a minimal safety margin. After bioassessment and analysis and review of the results, the samples should be disposed of to prevent accumulation of old and unusable samples. The storage area should be double locked and only authorized personnel be permitted to utilize the locked storage area. Chain of custody forms should be used to follow all samples.

16. Laboratory transport. A chain of custody form detailing sources and dates of sampling, descriptions of sample materials, and potential hazards should follow all samples. This form should be easy to use and cross referenced to a permanent record of the sample. As the sample is transported from individual to individual, a signoff should occur. The chain of custody form is signed off finally by the disposal officer after final disposal and then the form returned to the

file for permanent storage. All laboratory transport should follow a prescribed procedure from field, to transport, to laboratory storage, to preparation room where subsampling occurs and is recorded. The primary sample is returned to storage and the subsample is analyzed using bioassessment procedures. At successful termination of the bioassessment the waste material from the experimental assay and the primary sample should be transported to the disposal officer and then disposed of safely at a hazardous waste site.

17. Housekeeping. Good laboratory practices are the best guarantee of safety of personnel. Detailed procedures specifying handling, treatment, and disposal of samples and bioassay organisms should eliminate most potential problems. Prompt cleanup of all problems should occur to prevent more serious problems.

18. Laboratory facilities. Separate vacuum lines, water plumbing, and waste drainage must be provided. Careful labeling and isolation of facilities and maintenance equipment will minimize problems.

19. Emergency personnel. Potential problems that might occur and require emergency personnel should be carefully reviewed. Samples that do not require emergency personnel should be handled separately from those that might. If emergency personnel might be required, it is important to check with them in advance of such requirements and it is the

responsibility of the safety committee to insure that this process is followed.